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Effects of oxysterols on acyl-CoA: cholesterol acyltranferase

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Effects of oxysterols on acyl-CoA:cholesterol acyltransferase

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ABBREVIATIONS

acyl-CoA:cholesterol acyltransferase	ACAT
lecithin:cholesterol acyltransferase	LCAT
3-methyl-3-hydroxyl-glutaryl-CoA	HMG-CoA
20(S)-20,25-dihydroperoxy-5-cholesten-3ß-ol	HP-A
20(R)-20,25-dihydroperoxy-5-cholesten-3ß-ol	HP-B
20(S and R)-20,25-dihydroperoxy-5-cholesten-3ß-ol	HPs
25-hydroxycholesterol	25-OH
7α-hydroperoxycholesterol	7α-HP
phosphatidylcholine	PC
p-hydroxy-mecuribenzoate	PMB
phenylarsine oxide	PAO
dithiothreitol	DTT
2,3-dimercaptopropanol	DMP
N-methyl-mercaptoacetamide	MMA
enkephalinamide	EPA
methionine sulfoxide	Met(O)
methionine sulfone	$Met(O)_2$
cyanogen bromide	CNBr
deoxycholate	DOC

I. INTRODUCTION

Oxysterols produced from oxidation of cholesterol or biosynthetic precursors of cholesterol have a variety of biological activities. Some of these include cytotoxicity, atherogenicity, mutagenicity and carcinogenicity. In addition, several oxysterols are potent inhibitors of sterol biosynthesis in animal cells. A review of these topics was given by Smith and Johnson recently (1). The biological activities of oxysterols have been of particular concern with respect to human disease, such as atherosclerosis. Oxysterols are toxic to the three major cells of arterial wall, endothelial cells (2), smooth muscle cells (3), and fibroblasts (4), in vitro. Intravenous or gastric administration of oxysterols resulted in aortic lesions (5,6). Moreover, oxysterols have been identified in oxidized low-density-lipoprotein (LDL), which is a product from cell catalyzed oxidation (7). The presence of oxysterols may be related to the cytotoxicity of oxidized LDL. The atherogenicity of oxysterols was argued by Higley et al. (8) who compared the effect of feeding rabbits pure cholesterol and cholesterol oxidation products. They found that rabbits fed pure cholesterol exhibited more severe arterial lesions than those fed cholesterol-free oxysterols. In earlier experiments, we also found that a mixture of cholesterol autoxidation products was able to protect rabbits

against atherogenesis (9). Rabbits fed a diet containing 1% cholesterol developed much more severe aorta fatty streaks during an 8-week experimental period than those fed the same diet supplemented with the cholesterol oxidation mixture. Pre-reaction of the cholesterol autoxidation products with NaI completely abolished their effects on preventing atheroma formation. Accordingly, the active components in the mixture were suggested to be one or more cholesterol hydroperoxides.

A pair of epimeric cholesterol hydroperoxides were isolated from the mixture of cholesterol autoxidation products. Their structures were characterized as 20(S)-20,25-dihydroperoxy-5-cholesten-3β-ol (HP-A) and 20(R)-20,25-dihydroperoxy-5-cholesten-3β-ol (HP-B) (10). In preliminary experiments (11), we found that HP-A and HP-B were very effective in inhibition of cholesterol ester synthesis and accumulation by mouse peritoneal macrophages that were incubated with a cholesterol donor, acetylated low-density-lipoprotein (Ac-LDL). In cell-free membrane preparation, HPs inhibited mouse liver microsomal ACAT activity in a concentration dependent way similar to that which was observed in the intact cells. Moreover, HPs reduced the degradation rate of Ac-LDL by macrophages without causing an accumulation of intracellular lipoprotein. These results indicate that HPs inhibited macrophage cholesterol ester accumulation through at least two different mechanisms, suppression of cholesterol esterification and interruption of Ac-LDL metabolism.

In the present study, I further investigated the inhibitory effect of cholesterol hydroperoxides on ACAT activity. The potencies of a variety of oxysterols, synthetic or isolated from cholesterol autoxidation products, against ACAT were determined. A series of studies were performed to characterize the inhibitory mechanism of HPs. I examined the possibility that HPs might compete with cholesterol as substrate for the enzyme. I also determined the reactivity of HPs toward cysteine and methionine residues in protein. The chemical nature of ACAT inhibition was investigated with specific protein modifying agents and reducing agents. Finally, a possible mechanism of ACAT inhibition by HPs was proposed.

II. LITERATURE REVIEW

A. Purification and structural properties of ACAT

Acyl-CoA:cholesterol acyltransferase (ACAT) (EC.2.3.1.26) catalyzes the cholesterol esterification by utilizing cholesterol and long-chain fatty acyl-CoA as substrates. This enzyme is responsible for the major cholesterol ester synthesis in a variety of tissues under physiological conditions (see rev. 12). Despite its recognize importance, the structure and catalytic mechanism of ACAT are not well characterized. This is mainly due to the lack of purified enzyme. ACAT is a membrane protein that is located in the rough endoplasmic reticulum (13,14). Purification of this enzyme has been extremely difficult because of its instability in the presence of detergents. Moreover, after being extracted from the membrane, the enzyme has to be reconstituted into liposomes in order to measure its activity. A procedure for solubilization, partial purification, and reconstitution of pig liver ACAT was reported (15). The liver enzyme was extracted with 1.1% deoxycholate in combination with 1 N KCl and further purified by ammonium acetate fractionation and Sepharose column chromatography. The entire procedure produced a 150-fold increase in ACAT specific activity with 40% recovery of microsomal enzyme activity. Recently, an ACAT-candidate protein with 58 KDa

apparent molecular weight was identified from pig liver microsomes (16). In this report, the author only briefly outlined the scheme of purification, which included solubilization with 25 mM CHAPS, Concanavalin-A affinity chromatography, free flow isotachophoresis, iso-electric focusing, and Mono Q ion-exchange chromatography. The purified fractions were resolved by SDS-PAGE and blotted on a membrane. Then, ACAT activity was monitored by incubating the membrane strips with cholesterol-rich liposomes. Whether the 58 KDa protein is ACAT or some other esterifying enzyme, such as pancreatic cholesterol esterase or cholesterol esterase, awaits further investigation. The functional size of ACAT has been estimated by a radiation inactivation technique that does not require purified enzyme (17). Microsomal ACAT activity, measured by using labeled oleyl-CoA as substrate, decreased as a simple exponential function of radiation doses, leading to a target size of 170-180 KDa. When the size of microsomal acyl-CoA hydrolase was determined, it was found that this protein, approximately 46 kDa, was coupled to a large inhibitor, approximately 180 kDa. According to these observations, the authors proposed that acyl-CoA hydrolase is the acyl-CoA binding component and the inhibitor is the cholesterol-binding component of ACAT.

A systematic survey of the effects of chemical modification on ACAT activity was reported by Kinnunen et al (18). It was found that rabbit aortic and liver ACAT was

sensitive to sulfhydryl, histidyl, arginyl, and possibly tyrosyl modification agents. Base on the sensitivity toward a histidyl modification agent, diethyl pyrocarbonate, two distinct tissue subtypes of ACAT were identified. The apparent K_i of the liver ACAT was 1500 μM, compared to 40 μM for the aortic ACAT. The existence of two subtypes of ACAT was further confirmed in 14 different organs. The authors suggested that the heterogeneity of ACAT may contribute to the heterogeneous response of ACAT activity in different organs to cholesterol feeding. A detailed study of the effect of the thiol-modifying agent, p-mercuribenzoate, on ACAT activity indicated that there were at least two sulfhydryl groups influencing ACAT activity: one was at or near inhibitory binding site of coenzyme A, and another was important for cholesterol esterification (19). It was noted that inhibition of ACAT by modification of histidine and cysteine residues suggests the possible similarities of this enzyme to another cholesterol esterification enzyme, lecithin cholesterol acyltransferase (LCAT), which is responsible for plasma cholesteryl ester synthesis. LCAT has been shown to contain essential cysteine, histidine, and serine residues and a serine residue was possibly involved in the hydrolysis of lecithin but not in cholesterol esterification (20). Thus, ACAT and LCAT may share some common structural and mechanistic features.

B. Role of ACAT in aortic tissue, liver, and intestine

The role of ACAT in aortic tissue is of pathological interest. It has been considered that vascular ACAT activity may be a critical control point for cholesterol ester accumulation during atherogenesis (12). Aortic tissues from animals with experimental atherosclerosis have very high ACAT activity (21,22). Cultured macrophages are widely used as a model system to study the process of foam cell formation, a prominent feature of atherosclerotic lesions (23). It is found that incubation of macrophages with human Ac-LDL or ß-VLDL isolated from the plasma of cholesterol-fed dogs stimulated cellular synthesis and accumulation of cholesteryl ester by 20-160 fold (24). The accumulated cholesteryl ester in macrophages is stored in the form of cytosolic lipid droplets and undergoes a continuous cycle of hydrolysis and re-esterification, catalyzed by neutral cholesteryl ester hydrolase and ACAT (25). Because the re-esterification of cholesterol uses fatty acyl-CoA, which requires ATP for its synthesis, the process of hydrolysis and re-esterification is energy consuming. Such a futile cycle is speculated to contribute to the eventual necrosis of foam cells. The cholesterol esterification cycle can be interrupted by the presence of high-density lipoprotein (HDL), which releases un-esterified cholesterol from the cells (26). It has been demonstrated that the binding of HDL is reciprocally related

to ACAT activity (27,28). Incubation of cholesterol-laden macrophages with ACAT inhibitors, such as Sandoz 58.035, octimibate, and progesterone, resulted in a time- and dose-dependent elevation in HDL binding and HDL-mediated cholesterol efflux. Thus, the balance of cellular cholesterol in macrophages may be maintained by ACAT activity and the HDL-dependent cholesterol efflux. When ACAT activity is blocked the cholesterol level can be regulated by the number of HDL binding sites.

In liver, ACAT activity is also important in maintaining the homeostasis of cellular cholesterol. It is found that perturbation of cholesterol metabolism by feeding rats cholesterol, cholate, or an atherogenic diet increased the hepatic cholesterol concentration as well as ACAT activity (29). ACAT activity showed a positive correlation with changes in the concentration of un-esterified and esterified cholesterol. Inducing endogenous cholesterol synthesis by intra-gastric administration of mevalonolactone also enhanced hepatic ACAT activity. By regulating the ratio of un-esterified cholesterol to esterified cholesterol in liver, ACAT may also be a determinant of secretion of bile acid and lipoproteins. It has been reported that injection of the ACAT inhibitor, progesterone, into male rats caused an increase in biliary cholesterol output (30). The secretion rate of biliary cholesterol inversely correlated with the hepatic microsomal cholesteryl ester concentration and ACAT activity.

This result indicates that cholesterol esterification in liver and cholesterol secretion in the bile are coordinately regulated.

HMG-CoA reductase is a key enzyme in cholesterol synthesis (31), and it is also a microsomal enzyme. Generally, increasing microsomal cholesterol concentration would cause a reduction of HMG-CoA reductase activity and a stimulation of ACAT activity (32). But, these two enzyme activities are not always reciprocally related. For example, feeding rats cholestyramine, a bile acid sequestrant, decreased cholesterol and bile acid absorption, resulting in an increase in hepatic HMG-CoA reductase activity without affecting ACAT activity (21). And, experimental hypercholesterolemia induced by feeding hamster saturated fatty acid greatly suppressed hepatic ACAT activity and cholesterol esterification but did not have any effect on HMG-CoA reductase. In contrast, cholesterol feeding stimulated cholesterol esterification and inhibited the synthesis (33).

Intestinal ACAT activity is closely related to cholesterol absorption. In rat, rabbit, and human intestine, the highest ACAT activity is located in the jejunum and proximal ileum (34,35) where cholesterol and fat-soluble vitamins are mainly absorbed (36). Compound CL 277082 specifically inhibited ACAT in microsomes prepared from liver, adrenal, and intestinal mucosal cells and had no effect on incorporation of fatty acids into triacylglycerol and phospholipid. Feeding

rats cholesterol and this compound resulted in a profound reduction of plasma and hepatic cholesterol concentrations compared with the cholesterol-fed rats. When [^{14}C]-cholesterol was given to trace the fate of cholesterol, it was found that animals fed the ACAT inhibitor increased the excretion of neutral [^{14}C]-sterol in the feces (37). The regulation of cholesterol uptake and secretion by ACAT was demonstrated in the human intestinal cell line, CaCo-2. When cells were incubated with PD 128043, a competitive inhibitor of ACAT, the incorporation of oleate into cholesterol ester was blocked, as was the secretion of newly synthesized cholesteryl ester. In a chronic treatment, the inhibitor also suppressed the uptake of exogenous cholesterol, which was in the form of bile salt micelles (38).

C. Regulation of ACAT activity

1. Substrate availability

The fact that microsomal ACAT activities from a variety of tissues can be stimulated by exogenous cholesterol indicates that under normal physiological conditions, the cholesterol substrate pool for ACAT is not saturated. Thus, ACAT activity is limited by the amount of cholesterol available in the substrate pool. Because 90% of free cholesterol in cells is located in the plasma membrane and, possibly, associated with sphingomyelin (39), intracellular cholesterol transport is

important in determining the ACAT activity. Treating cultured fibroblasts with sphingomyelinase caused a rapid release of plasma cholesterol into the intracellular pool. The influx of cholesterol resulted in a 15-fold increase in the rate of cholesterol esterification (40). In cell-free membrane preparations, cholesterol transfer between heterogeneous microsomal vesicles (endoplasmic reticulum and plasma membrane) also affects ACAT activity. For example, ACAT activity increased in a time- and temperature-dependent way, even when microsomes were preincubated in the absence of exogenous cholesterol (41). The addition of exogenous cholesterol in the form of phospholipid liposomes or mixed micelles with detergents effectively stimulated microsomal ACAT activity (41,42,43). The exogenous cholesterol quickly equilibrates with endogenous cholesterol. In one example, about 40-50% of newly synthesized cholesteryl ester was found to be derived from exogenous substrate (42).

Some cytosolic factors may facilitate the intervesicular cholesterol transport. Inclusion of cytosol in the incubation of microsomes with cholesterol-rich liposomes accelerates the cholesterol transfer and enhances the ACAT activity (44,45). One candidate for such a cytosolic protein is purified from rat liver and named sterol carrier protein 2 (SCP_2). In vitro study demonstrates that SCP_2 caused a striking increase in microsomal ACAT activity as measured by using exogenous added [^{14}C]-cholesterol or [^{14}C]-oleoyl-CoA (35) . Whether this

protein plays a physiological role in intracellular cholesterol transfer is unclear.

2. Covalent modification

ACAT is possibly regulated by phosphorylation/dephosphorylation as are two other important enzymes in cholesterol metabolism, 3-hydroxyl-3-methyl-glutaryl-CoA reductase (46) and 7α-hydroxylase (47). Suckling et al (48,49) reported that incubation of microsomes prepared from rat liver or intestinal epithelial cells with the 100,000xg supernatant in the presence of ATP/Mg_{++} and NaF resulted in an increase in ACAT activity. The activation was dependent on the preincubation time and ATP concentration. When cholesterol-rich liposomes were included in the pre-incubation mixture, ACAT activity was further increased. Because the activation by ATP-dependent mechanism and by the addition of exogenous cholesterol were additive, the authors suggested that ACAT may be regulated by both substrate supply and (de-)phosphorylation. In another experiment reported by Mitropoulos and Venkatesan (41), microsomes were incubated with liposomes containing [^{3}H]-cholesterol in the presence or absence of cytosol. In either case, ACAT activity increased with an increase in preincubation time and temperature. The presence of cytosol significantly enhanced the rate of cholesterol transfer from liposomes to microsomes. The elevated cholesterol transfer was abolished if NaF was

included in the preincubation mixture. Thus, the intervesicular cholesterol transfer may be modulated by phosphorylation/dephosphorylation and contribute to the change of ACAT activity.

3. Possible physiological inhibitors and activators

The existence of a short-lived ACAT inhibitor has been postulated by two different research groups. Studies with Chinese hamster ovary cells demonstrated that the addition of a protein synthesis inhibitor, cycloheximide, caused an increase in cellular ACAT activity measured in cells incubated in lipoprotein-depleted medium. The ACAT activation increased with time and reached a maximum at 6-8 hr. Incubation of cells with lipoprotein-supplemented medium, 25-hydroxycholesterol, or an excess of mevalonate all increased ACAT activity and abolished the stimulatory effect of cycloheximide (50,51). The authors suggested that the activation by cycloheximide is possibly due to the depletion of a short-lived (turn-over rate: 6-8 hr) inhibitor of ACAT. An increase in exogenous cholesterol or in endogenous cholesterol synthesis might either block the activity or accelerate the degradation of this inhibitor. On the other hand, Tabas and Boykow (52) observed that incubation with LDL did not cause an increase in ACAT activity nor cholesterol ester accumulation in peritoneal macrophages, despite both substantial uptake and degradation of LDL in these cells.

When peritoneal macrophages were treated with protein synthesis inhibitors, cycloheximide, puromycin, or actinomycin D, the LDL-induced whole cell ACAT activity and cholesterol ester accumulation were 10-fold higher than that seen in the LDL-incubated cells. The ACAT activation was detected 4 hr after the addition of protein synthesis inhibitors and disappeared 4 hr after the removal of inhibitors. ACAT assay with cell-free membrane preparations from cycloheximide treated cells or control cells showed that the activation effect on microsomal cholesterol esterification was much less than on the intact cells. Thus, the maximal ACAT activation by protein synthesis inhibitors appeared to require the intact cell. The authors suggested that another possible mechanism for the short-lived inhibitor was to affect the delivery of cholesterol to ACAT rather to inhibit the enzyme itself.

25-Hydroxycholesterol has been shown to stimulate cholesterol esterification in a variety of intact cells that are incubated in media containing exogenous cholesterol (see rev. 1). But, in one example, the ACAT activity measured in microsomes isolated from 25-OH treated macrophages was no different from that from the control cells (53). Moreover, the stimulatory effect of 25-OH on microsomal ACAT was decreased if microsomes were preincubated with cholesterol-rich liposomes. And, the stimulatory effect was inversely related to the microsomal cholesterol content (54). The molecular mechanism of ACAT activation by 25-OH is not clear.

It has been proposed that the oxysterol may either affect cholesterol delivery to the enzyme substrate pool or it may interact with a regulatory site on the enzyme (54).

III. EXPERIMENTAL

A. Materials and equipment

Aprotinin, pepstatin, leupeptin, antipain, benzamidine, phosphate buffered saline, dioleoyl-L-α-phosphatidylcholine, cholestyramine, glutathione, glutathione disulfide, iodoacetic acid, 1-fluoro-2,4-dinitrobenzene, p-hydroxy-mercuribenzoate, 5,5'-dithio-bis-(2-nitrobenzoic acid), phenylarsine oxide, 2,3-dimercaptopropanol, dithiothreitol, leucine enkephalinamide, methionine enkephalinamide, and cyanogen bromide were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium cholate, sodium deoxycholate, bovine serum albumin, and calmodulin were obtained from Calbiochem Co. (San Diego, CA). Reagents used for electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA). [^{14}C]-Oleoyl-CoA and [^{3}H]-oleate were purchased from New England Nuclear Corp. (Boston, MA). Silica gel (40-140 mesh) was purchased from J. T. Baker Inc. (Phillipsburg, NY). Hexane and isopropanol were purchased from Fisher Scientific (Pittsburgh, PA). Scintillation fluid, Ecolume, was supplied by ICN Biomedicals Inc. (Costa Mesa, CA). Licrosorb PR-18 and 3-amino-propyl HPLC columns were supplied by E. Merck (Darmstadt) and Custom LC Inc. (Houston, TX), respectively. Cholesterol purchased from Nutritional Biochemical Inc. (Cleveland, OH) was recrystallized from methanol three times before use. 20(S and

R)-20,25-dihydroperoxy-cholest-5-ene-3ß-ol (HP-A and HP-B), 25-hydroperoxycholesterol, 20(S and R)-20-hydroperoxycholesterol, and 7α-hydroperoxycholesterol were prepared as described by Tipton et al. (10). The following hydroperoxides were provided by Dr. M. Ranasinghe, Department of Chemistry, Iowa State University, Ames, Iowa: 20(S and R)-20-hydroperoxycholest-5-ene-3ß,25-diol (HP-A' and HP-B'), cholest-5-ene-3ß, 20(S and R), 25-triol (Triol-A and Triol-B), 20(S and R)-20,25-dihydroperoxy-24-nor-cholest-5-ene-3ß-ol (HP-11A and HP-11B), 20(S and R)-20,26-dihydroperoxy-homocholest-5-ene-3ß-ol (HP-19A and HP-19B), and 1-(1',5'-dihydroperoxy-1,5-dimethylhexyl)indan (HP-72A and HP-72B). N-Methyl-mercaptoacetamide was prepared by reaction of ethylmercaptoacetate with methylamine as described (55).

Equipment used included a Tissumizer homogenizer (Tekmar Co., Cincinnati, OH), Sorvall RC-5B centrifuge (Du Pont Instruments, Newtown, CN), Beckman L8-80 ultracentrifuge (Beckman Instruments Inc., Fullerton, CA), Spectra/Pro 10-well microdialyzer (Spectrum medical industries Inc., Los Angeles, CA), Sonicator Bransonic 12 (Branson Cleaning equipment Co., Shelton, CN), bench-top centrifuge, Centra-Mat (International Equipment Co., Needham Heights, MA), and Hoefer mini-gel apparatus (Hoefer Inc., San Francisco, CA). For glutathione analysis, a HPLC system consisting of one SP8700 pump (Spectra Physics Co.,), one ISCO V^4 detector (ISCO Inc., Lincoln, NE), and one HP 3390A integrator (Hewlett

Packard Co., Avondale, PA) was used. Another HPLC system consisting of Dural Pump 2350, one V^4 detector, Chem Research data management and system control from ISCO Inc. was used for peptide analysis. Scintillation counter, Beta-trac 6895, was purchased from Tm Analytic Co. (Elk Grove Village, IL).

B. Methods

1. ACAT Assay

Preparation of mouse liver microsomes Male Swiss Webster mice were injected intraperitoneal with lipopolysaccharide 4 days before experiments. Mouse livers were perfused with ice cold phosphate-buffered saline and homogenized with Tissumizer in a buffer containing 50 mM Tris-HCl, 1 mM EDTA, pH 7.7 (buffer A), and a mixture of protease inhibitors, including: aprotinin, pepstatin, leupeptin, antipain, and benzamidine. The concentrations of aprotinin, pepstatin, leupeptin, and antipain were 1 μg/ml and that of benzamidine was 17 μg/ml (56). The liver homogenate was centrifuged at 10,000xg (Sorvall SS 34 rotor, 9.1K rpm), at 4°C for 30 min, and the supernatant was then centrifuged at 100,000xg (Beckman 50 Ti rotor, 39K rpm), at 4°C for 1 hr. The pellet was washed with buffer A and centrifuged again. The microsomal pellet was collected and re-suspended in buffer A containing protease inhibitors to a final concentration approximately 20-28 protein mg/ml.

Extraction of microsomal ACAT Routinely, microsomal ACAT was extracted with 1.5% deoxycholate (DOC) combined with 1 N KCl. Microsomes (0.5 ml) were mixed with 50 mg KCl and 74 µl buffer A prior to the addition of deoxycholate. One hundred microliters 10% deoxycholate was added to the microsomes dropwise with slow stirring. The mixture was stirred for another 30 min at 4°C and then centrifuged at 160,000xg (Beckman SW55 rotor at 41K rpm) for 1 hr at 4°C. The supernatant was collected and diluted with an equal volume of 1% sodium cholate. The protein content in the microsomal extract was approximately 5-8 mg/ml. The extract was stored at 4°C and used within 2 days. ACAT activity in the microsomal extract was detected either after 50-fold dilution or after dialysis against buffer A.

Preparation of lipid/detergent mixture Cholesterol and dioleoyl-phosphatidylcholine dissolved in chloroform were added to a 13x75mm disposable test tube. Solvent was vaporized with a stream of nitrogen. A few drops of diethyl ether were added to the lipid layer and the solvent was evaporated again. The addition and evaporation of diethyl ether were repeated three times, so that the solvent was completely removed. To every 10 mg PC, one milliliter of 0.2% sodium cholate in buffer A was added. The mixture was flushed with nitrogen, and the test tube was sealed with a septum. The lipid/detergent mixture was then sonicated in a Bransonic sonicator to m aximal clarity, usually 20-30 min.

Reconstituted liposomes Liposomes were prepared by either a dialysis method (method A) (15) or the cholestyramine method (method B) (57). Method A: Twenty microliters of microsomal extract was diluted with 180 μl lipid/detergent mixture and dialyzed against a constant flow (2.5 ml/min) of buffer A into a 10-well microdialyzer at 4°C for 3 hr. Reconstituted liposomes formed by this procedure were diluted 1:5 with buffer A before ACAT assay. Method B: Cholestyramine was washed twice with buffer A. To each 0.5 ml lipid/ detergent mixture, twenty five minigrams cholestyramine was added. After vortexing, the mixture was centrifuged in the Centra-Mat centrifuge for 10 min at room temperature. The supernatant was treated with 25 mg cholestyramine and centrifuged again. Finally, the supernatant was centrifuged to remove trace amounts of insoluble materials. Nine portions of these liposomes were mixed with one portion of microsomal extract. The mixture was allowed to stand at room temperature at least 30 min before ACAT activity was measured.

Assay procedure The assay procedure for ACAT described by Chang et al (15) was followed with minimal modification. An aliquot of 160 μl diluted microsomes or reconstituted liposomes containing 15-200 μg protein was used for each assay. The enzyme solution was preincubated 10 min at 37°C prior to the addition of 40 μl substrate mixture. The substrate mixture consisted of 250 μM [^{14}C]-oleoyl CoA (25 dpm/pmole) and 0.5 mg fatty acid free bovine serum albumin

dissolved in buffer A. The assay was terminated after 5 min by the addition of 2 ml hexane/isopropanol (3/2, v/v). An aliquot of cholesterol-[^{3}H]-oleate (about 1000 cpm) dissolved in absolute ethanol was introduced into each sample to estimate the recovery. Cholesteryl ester was isolated from the lipid extract by silica gel chromatography as described by Chautan (58). The cholesteryl ester eluate was collected in a 7-ml polyvinyl ester scintillation vial. After evaporation of solvent, cholesteryl ester was redissolved in 0.2 ml hexane/diethyl ether (98/2, v/v), followed by the addition of 5 ml Ecolume counting fluid. The radioactivity of cholesteryl ester was measured by a liquid scintillation counter, Beta-trac 6895, with channel A setting from 000 to 240 and channel B from 240 to 999.

2. Reaction of ACAT with oxysterols and related compounds

Reactivity of HP analogs Microsomes (100-200 μg/assay) were preincubated with 31 μM oxysterols or other related compounds at 37°C for 15 min. Then, the ACAT assay was initiated with the addition of [^{14}C]-oleoyl-CoA. In the control experiment, an equal volume of ethanol was added to the microsomes during the preincubation instead of the ethanolic solution of the compound being tested.

Effect of 25-hydroxycholesterol The effect of 25-OH on ACAT activity requires a longer incubation time. Therefore, a 60 min incubation was adopted in this case. Thus, 25-OH was

added to microsomes or reconstituted liposomes and the mixture was incubated for 60 min at 37°C before the ACAT activity was determined.

3. Oxidation of model peptides and protein

Oxidation of glutathione One volume of ethanol or 10 mM ethanolic HP-B was mixed with four volumes of 1 mM glutathione or glutathione disulfide dissolved in buffer A. The reaction was allowed to proceed at 37°C for 8 hr. After lyophilization, the reaction products were reacted with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene as described (59), and the derivatives were analyzed with HPLC (60) with a 3-amino-propyl column.

Oxidation of enkephalinamide Leucine and methionine enkephalinamides (Leu-EPA and Met-EPA) were dissolved in buffer A to 1 mg/ml (about 1.7 mM). An equal volume of 10 mM ethanolic cumene hydroperoxide (cumene-HP) or HP-B was added to the peptide solutions, and the mixtures were incubated at 37°C. At the stated times, aliquots of the reaction mixture were withdrawn and analyzed with an ISCO HPLC system. The peptides were eluted with a linear gradient solvent program: solvent A was water containing 0.1% trifluoroacetic acid (TFA), and solvent B was 75% acetonitrile in water containing 0.1% TFA. Solvent B was increased from 25 to 40% during the first 15 min, and at 40% for another 10 min. The flow rate was 0.7 ml/min. Under these conditions, Leu-EPA, Met-EPA,

and methionine sulfoxide-EPA (Met(O)-EPA) were eluted at 18.4, 15.3, and 7.8 min, respectively. When the flow rate was increased to 0.85 ml/min, Met-EPA, Met(O)-EPA, and methionine sulfone-EPA ($Met(O)_2$-EPA) were eluted at 13.0, 6.0, and 7.2 min, respectively.

Met-EPA and its oxidized derivatives were collected from HPLC. After evaporation of the solvent, the dry peptides were analyzed by mass spectroscopy. The peptides were first desorbed from a glycerol matrix and then ionized by fast atom bombardment.

Oxidation of calmodulin Fifty microgram samples of calmodulin (1 mg/ml in water) were added to microcentrifuge tubes containing 0.5 μl ethanol, 100 mM ethanolic HP-B or Triol-B, or 200 mM 7α-HP. The mixtures were incubated at 37°C for 1 hr followed by the addition of an equal volume of cyanogen bromide (2 or 20 mg/ml in 70% formic acid). The cyanogen bromide reaction proceeded in the dark at room temperature for 40 hr (61). At the end of the reaction, the mixture was diluted 4-fold with water and then lyophilized.

The lyophilized calmodulin fragments were rehydrated with water and analyzed with SDS-PAGE. A Hoefer mini-gel apparatus was used to perform the electrophoresis. Mini-polyacrylamide slab gels (15%) were prepared according to Laemmli's procedure (62). Protein bands were visualized by staining with Coomassie blue G.

4. Effects of thiol-blocking reagents and reducing agents on hydroperoxide-treated ACAT activities

Modification of ACAT with thiol-blocking reagents A stock solution of 4 mM p-hydroxy-mercuribenzoate (PMB) was prepared by dissolving the compound in buffer A. The solution was stored in the dark at room temperature. Microsomes (5 mg/ml) were incubated with varied concentrations of PMB at 37°C for 10 min, followed by a 10-fold dilution with buffer A with or without 1.3 mM dithiothreitol. ACAT activity was measured after 10 min incubation.

Varied amounts of phenylarsine oxide (PAO) dissolved in ethanol were added to microsomes, and the reaction was allowed to proceed for 30 min at 37°C . The concentration of ethanol in the incubation mixture was 1%. The mixture was diluted 8-fold with buffer A with or without dithiothreitol or 2,3-dimercaptopropanol. ACAT activity was determined 30 min after the dilution.

In the cases of double treatment, HP-B was added to PAO-treated microsomes to a final concentration of 100 μM, and the microsomes were incubated for 15 min before dilution. ACAT activity was measured 30 min after dilution. In control experiments, ethanol was used to replace the ethanolic solution of PAO or HP-B.

Activation of HP-B-treated ACAT with thiol reducing agents Sodium borohydride ($NaBH_4$), 2,3-dimercaptopropanol (DMP), and dithiothreitol (DTT) were dissolved in buffer A that was

flushed with N_2. A 50% N-methyl-mercaptoacetamide (MMA) stock solution was prepared and neutralized by 1 N NaOH. Microsomes (2-4 mg protein/ml) were reacted with 100 μM HP-B at 37°C for 15 min before the addition of reducing agents. The HP-B-treated microsomes were either directly diluted with buffer A containing reducing agents or reacted with an equal volume of reducing agents and then diluted with buffer A. Both short term, 15-30 min, and long term, 3 hr, incubations of microsomes in the presence of reducing agents were performed. Then, the ACAT activity was determined.

IV. RESULTS

A. ACAT assay

1. Enzyme solubilization

A two-step extraction of pig liver microsomal ACAT has been developed by Doolittle and Chang (15). Accordingly, microsomes were extracted with 0.5% deoxycholate combined with 1 M KCl, and the insoluble portion was re-extracted with 1.1% DOC. They then measured the ACAT activities in the 50-fold diluted DOC extracts. By this procedure, they were able extract 86% of the microsomal ACAT activity into the 1.1% DOC soluble fraction, and the enzyme specific activity was increased by 11-fold. I attempted to apply this extraction method to mouse liver microsomes, but found that mouse ACAT was very soluble in 0.5% DOC. As can be seen in Table 1, the 0.5% DOC extract contained 73% of total microsomal ACAT activity. The specific activity of ACAT in the extract was 1.8-fold of that in the original microsomes. ACAT remaining in the pellets from the first extraction was completely solubilized with 1.5% DOC. ACAT was stable through the whole procedure of the two-step extraction. The sum of enzyme activities of $S_{0.5\%}$ and $S_{1.5\%}$ was equal to the total enzyme activity in the original microsomes. Because of the solubility of ACAT in 0.5% DOC, the routine preparation of microsomal extract was done by directly extracting microsomes

Table 1. Extraction of ACAT from mouse liver microsomes. Microsomal ACAT was extracted with 0.5% DOC in the presence of 1 M KCl. The insoluble portion was resuspended in 1 M KCl and re-extracted with 1.5% DOC. $S_{0.5\%}$ and $S_{1.5\%}$ represent the soluble fractions. These fractions were dialyzed before ACAT activities were measured. $P_{0.5\%}$ and $P_{1.5\%}$ are the insoluble fractions. Their enzyme activities were measured after resuspending the pellets in buffer A. Each value is the mean of two determinations.

Fractions	ACAT activity pmole/min/mg	total activity pmole/min	percent of yield
Microsomes	120	5256	100
$S_{0.5\%}$	219	3836	73
$P_{0.5\%}$	153	1420	27
$S_{1.5\%}$	383	1436	27
$P_{1.5\%}$	44	157	3

with 1.5% DOC in the presence of 1 M KCl. Microsomal extract prepared in this way contained ACAT with specific activity 1.3- to 2.6-fold of that in the original microsomes. Unlike Doolittle's procedure, the measurement of ACAT activity shown in Table 1 was performed after dialysis of the detergent-soluble fraction for 3 hr against buffer A. ACAT was found to be much more active if DOC was removed from the microsomal extract by dialysis than if it was simply diluted. For example, ACAT activity was 316 pmole/min/mg in a dialyzed microsomal extract compared to only 58 pmole/min/mg in a 50-fold diluted extract.

2. Effect of cholesterol concentration on the inhibition of ACAT by HPs

The fact that HP-A and HP-B inhibited ACAT activity in the intact macrophages and liver microsomes in similar dose-dependent manners suggests that these two compounds are not distinguishable by the enzyme. Therefore, HP-A and HP-B were used alternatively in the following experiments. The effect of cholesterol concentration on ACAT activity was investigated in liposomes in which the lipid compositions were defined. In one experiment, HP-A (50 or 127 pmole HP-A per 2 mg phosphatidylcholine) was added to a lipid/detergent mixture, and the reconstituted liposomes were prepared according to Method A described in Experimental. The ratio of HP-A to total sterols in liposomes was varied from 3 to 18% or 7.5 to

45%. The result is shown in Figure 1. Because ACAT is distributed in the lipid bilayer, I express the concentration of cholesterol as mole fraction with respect to the total lipid in liposomes. In the absence of HP-A, ACAT activity increased with the increase of cholesterol concentration in a sigmoidal way, and reached the maximum at 0.3 mole fraction of cholesterol. The lowest cholesterol concentration, 0.015 mole fraction, came from the endogenous cholesterol in the microsomal extract. ACAT activity in these liposomes was referred to the basal level. In the presence of 37 μM HP-A, ACAT activity did not vary with cholesterol concentration and was only 37% higher than the basal level from 0.1 to 0.4 mole fraction cholesterol. With 91 μM HP-A, ACAT activity did not respond to the changes of cholesterol concentration and remained at the basal level. In another experiment, reconstituted liposomes were preformed according to the method B by which lipid concentrations in the assay solution was 6.4-fold more concentrated than that in the previous experiment. As shown in Figure 2, reconstituted ACAT activity was dependent on the cholesterol present in the liposomes. The maximal activity was reached at 0.4 mole fraction and decreased at 0.6 mole fraction of cholesterol. The addition of 31 μM HP-B to liposomes greatly suppressed the enzyme activity. Even when the cholesterol mole fraction was 0.4, giving a very high ratio of total sterol to HP-B, 325, cholesterol was not able to protect ACAT from inhibition by HP-B.

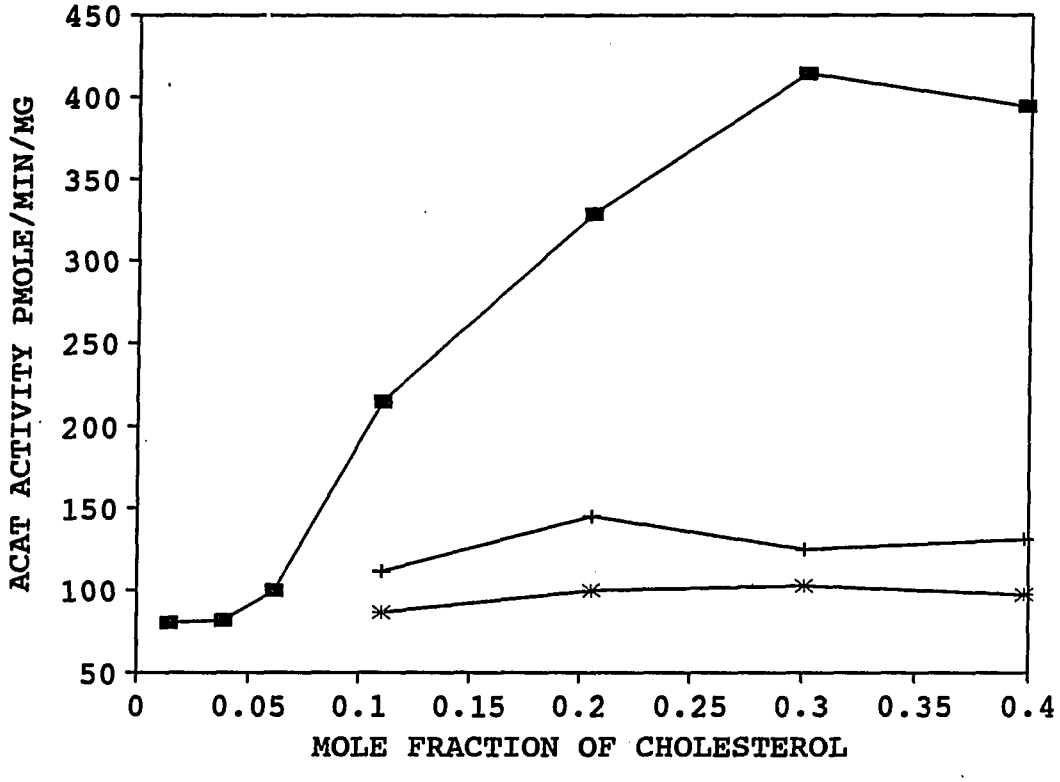


Figure 1. Effect of cholesterol mole fraction on the inhibitory effect of HP-A on ACAT activity. Lipid/detergent mixtures with/without HP-A were used to prepare the reconstituted liposomes. Cholesterol concentrations in the assay solution were 233,487, 814,and 1250 μM where mole fractions were 0.11, 0.21, 0.30, and 0.40. Control (■) had no HP-A. Otherwise, liposomes contained either 37 (+) or 91 (*) μM HP-A.

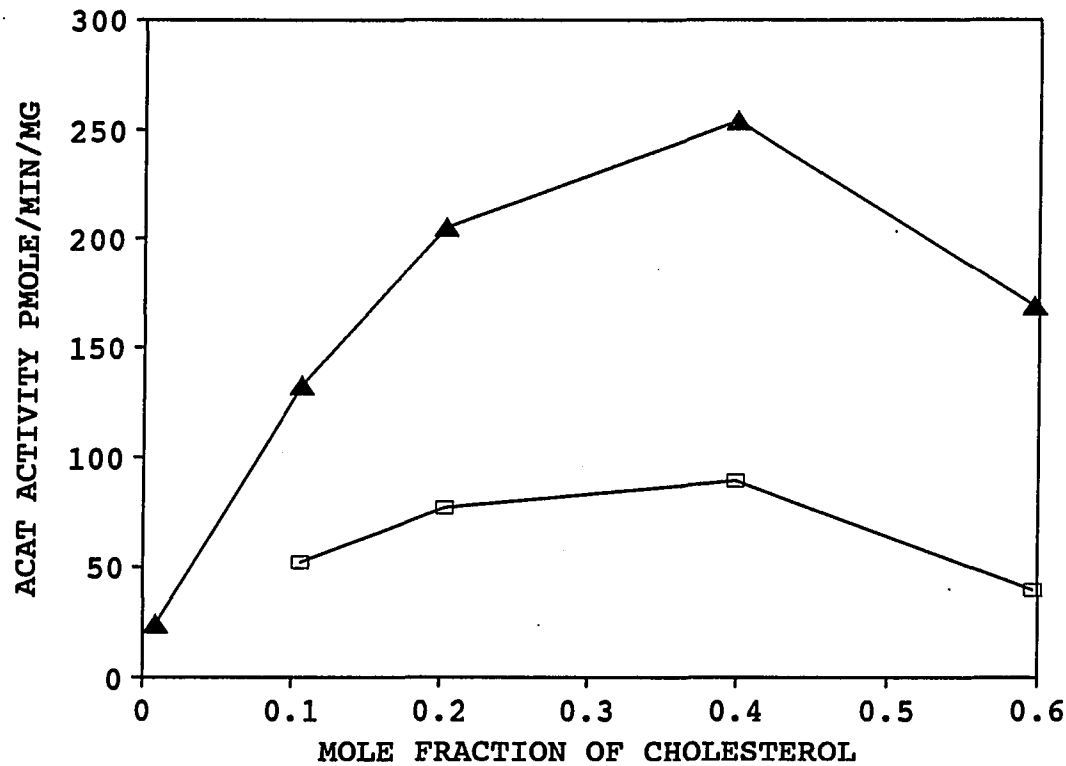


Figure 2. Effect of HP-B on ACAT activity in preformed liposomes. HP-B 25 μM (□) was added to the pre-formed reconstituted liposomes (prepared by Method-B). In the assay solutions, cholesterol concentrations were 1.4, 3.1, 5.2, 8.1, and 18 mM where its mole fractions were 0.11, 0.20, 0.40 and 0.60. In control group (▲), ethanol was added to replace HP-B.

B. Reaction of ACAT with oxysterols and related compounds

1. Effect of oxysterols on ACAT activity

Numerous cholesterol hydroperoxides, such as 7(α and ß)-HP, 20(R and S)-HP, and 25-HP, have been found in the mixture of air oxidized cholesterol (63,64). I compared their activities against ACAT. The results are shown in Figure 3. When microsomes were challenged with 31 μM of each hydroperoxide for 15 min, only HP-A and HP-B exerted potent inhibition of ACAT activity. This concentration of HP-B has been shown to cause maximal ACAT inhibition (11). The inhibitory power of the hydroperoxides in sequence is: HP-A, HP-B >> 20(S or R)-HP > 25-HP, 7α-HP. Several analogs of HP-A and HP-B have been synthesized by Dr. M. Ranasinghe and their structures are shown in Figure 4. One pair of epimers are named HP-A' and HP-B' for their structural similarity to HP-A and HP-B. Instead of having two hydroperoxyl groups, HP-A' and HP-B' contain only one hydroperoxyl group at C-20 and one hydroxy group at C-25. As shown in Table 2, the replacement of a hydroperoxyl group by a hydroxyl group at C-25 greatly diminished the inhibitory effect. HP-A' and HP-B' affected ACAT activity to a similar extent as the other monohydroperoxides, 20-HP and 25-HP did. Triol-A and Triol-B contain no hydroperoxyl group but nevertheless inhibited ACAT slightly. In Table 3, the potency of 62 μM HP-B' or cumene-HP was compared with that of 31 μM HP-B. As shown, ACAT activity

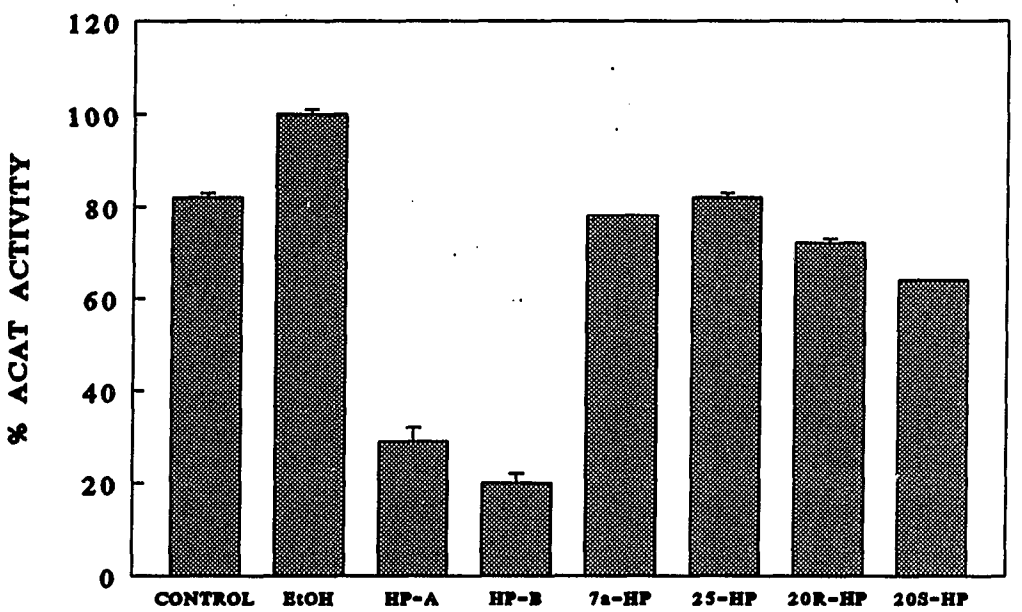


Figure 3. Effect of different cholesterol hydroperoxides on ACAT activity. Microsomes were incubated with the indicated hydroperoxides for 15 min at 37°C before the ACAT activities were measured. Control: microsomes with no treatment; EtOH: only ethanol was added. The enzyme activities measured in microsomes with different treatments are compared to that in the ethanol treated-microsomes.

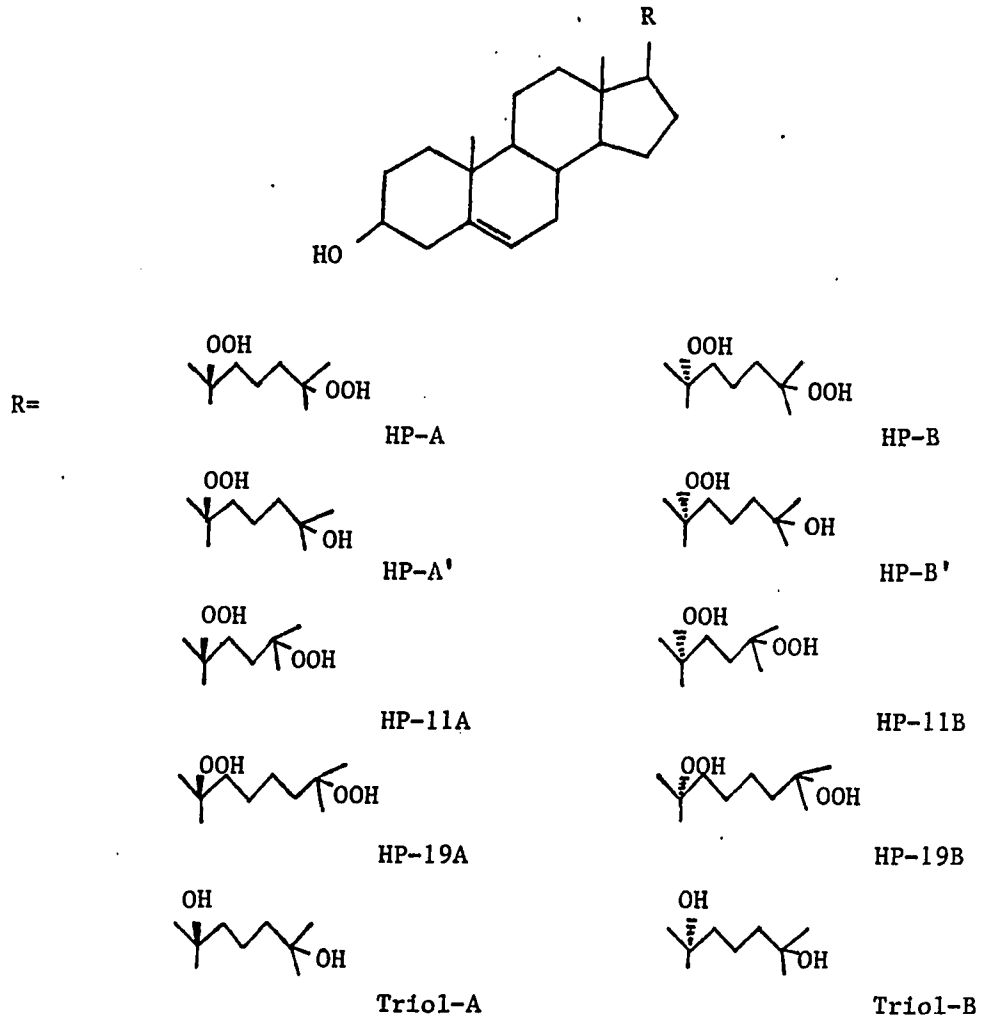


Figure 4. Structures of the synthetic HPs analogs

Table 2. Effect of reduction of hydroperoxy groups of HP-A and HP-B on their potencies as inhibitors of ACAT. Microsomes were incubated with 31 μM sterol for 15 min at 37°C before the ACAT activities were determined. Each value is the mean ± s.d. of two determinations.

Treatment	ACAT activity pmole/min/mg	percent of control
Control	463 ± 50	100
HP-B	167 ± 15	36
HP-A'	422 ± 10	91
HP-B'	387 ± 22	84
Control	763 ± 8	100
HP-B	183 ± 4	24
Triol-A	581 ± 40	76
Triol-B	555 ± 2	73

Table 3. Comparison of ACAT-inhibitory effects of mono- and di-hydroperoxides. Microsomes were incubated with different hydroperoxides for 15 min at 37°C prior to the ACAT assay. Each value is the mean ± s.d. of two determinations.

Treatment	ACAT activity pmole/min/mg	percent of control
Control	415 ± 1	100
31 μM HP-B	82 ± 0	20
62 μM HP-B'	351 ± 4	85
62 μM Cumene-HP	265 ± 6	64

was less affected by theformer two compounds than by the latter one even though the concentration of hydroperoxyl groups in these experiments was the same. HP-11(A and B) and HP-19 (A and B) are cholesterol dihydroperoxides with side chains either one carbon shorter or one carbon longer than the HP-A and HP-B. Their activities against ACAT are shown in Table 4. Decreasing the distance between the two hydroperoxyl groups by deletion of a methylene group decreased the potency slightly. Increasing the distance by one methylene decreased the potency (HP-19B) or left it almost unchanged (HP-19A). The side chains of HP-72A and HP-72B resemble that of HP-A and HP-B, and are connected to an indan rather than the tetracyclic structure of cholesterol. The indan hydroperoxides were found to be as good ACAT inhibitors as were HP-A and HP-B (Table 4). The corresponding indan diols were inactive.

2. Effect of 25-OH on ACAT activity

25-Hydroxycholesterol (25-OH) has been shown to stimulate ACAT activity in intact cells as well as in microsomes (see Discussion). Here, a time course study of ACAT stimulation by 25-OH was performed, and the result indicated that ACAT stimulation was time dependent. Figure 5 shows that the enzyme activity was increased by 18% after 1 hr incubation with 50 μM 25-OH, whereas a ten min incubation has no detectable effect. The stimulatory effect was more

Table 4. The activities of synthetic hydroperoxides against ACAT. Microsomes were incubated with hydroperoxide for 15 min at 37°C before the ACAT assay was started. The concentration of the sterols and indan derivatives was 31 μM. Each value is the mean ± s.d. of two determinations.

Treatment	ACAT activity pmole/min/mg	percent of control
Control	703 ± 1	100
HP-B	335 ± 23	48
HP-11A	457 ± 7	65
HP-11B	487 ± 19	69
HP-19A	385 ± 11	55
HP-19B	592 ± 28	84
HP-72A	366 ± 23	52
HP-72B	391 ± 10	56
Control	763 ± 8	100
HP-B	183 ± 4	24
HP-72A	156 ± 12	20
Dihydroxyl-72	716 ± 17	94

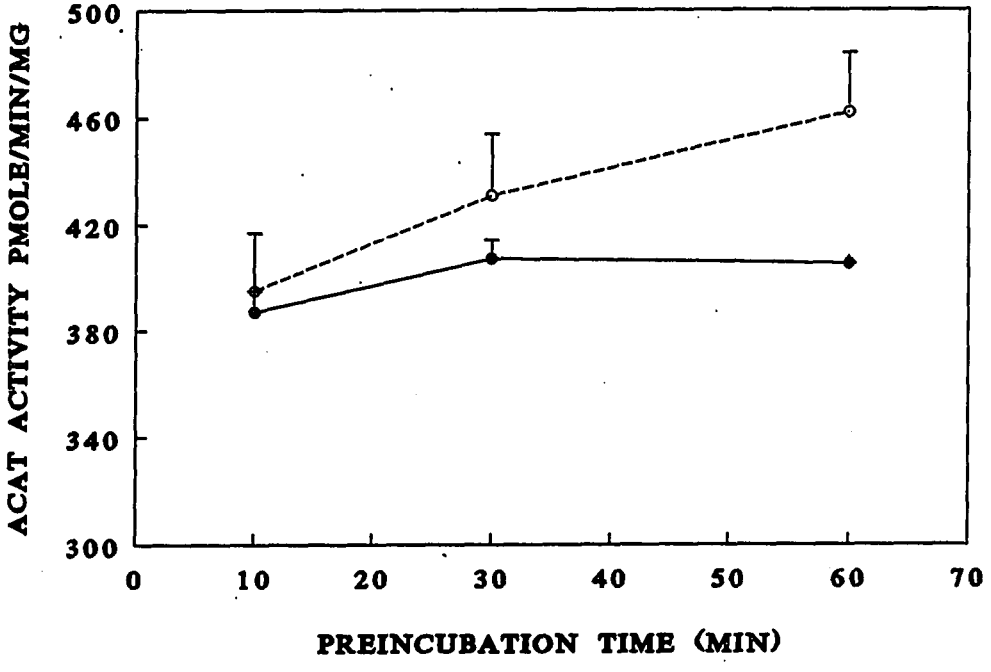


Figure 5. Time-dependent activation of ACAT activity by 25-hydroxycholesterol. Microsomes were incubated with (O) or without (●) 31 μM 25-OH at 37°C. At the indicated times, an aliquot of the incubation mixture was withdrawn for ACAT assay.

profound with freshly prepared microsomes than with pre-frozen or heat-treated microsomes. As shown in Table 5, the process of freezing and thawing did not affect ACAT activity; however, it had a great influence on the stimulatory effect of 25-OH. While 25-OH increased the enzyme activity by 42% in the freshly prepared microsomes, the pre-frozen microsomes only responded by 30% of activation. ACAT was labile to the heat treatment, which caused a loss of one third of activity in 3 min at 60°C. In addition, ACAT became less sensitive to 25-OH after the heat treatment.

The effect of 25-OH was further investigated in the reconstituted liposomes. The result is shown in Figure 6. 25-OH at 31 μM enhanced ACAT activity in liposomes containing 0.02 to 0.11 mole fraction of cholesterol and slightly decreased the enzyme activity in liposomes with 0.6 mole fraction of cholesterol. In the presence or absence of 25-OH, the enzyme activity showed a sigmoidal response to the increase in cholesterol concentration.

Figure 7 shows the result of challenging the stimulatory effect by HP-B. In this experiment, microsomes were sequentially incubated with 25-OH then HP-B or vice versa. With 31 μM 25-OH, ACAT activity was increased by 13% after 75 min incubation. At the same concentration, HP-B inhibited 57% of the enzyme activity. Incubation of 25-OH treated microsomes with HP-B totally destroyed the stimulatory effect, and furthermore, the enzyme activity was decreased to the same

Table 5. Effect of 25-hydroxycholesterol on microsomal ACAT activity after heating or freezing. Microsomes were prepared according to the procedure described in Experimental. A portion of microsomes was quick-frozen in dry ice-cooled ethanol, and ACAT activity was determined after thawing the microsomes in a 22°C water bath. Another portion of microsomes was incubated at 60°C for 3, min and the precipitate was removed by centrifugation. All microsomes were incubated with 31 μM of the oxysterol for 1 hr before ACAT activity was measured. Each value is the mean ± s.d. of two determinations. The degree of activation is shown in the parenthesis.

Microsomes	ACAT activity	
	-25-OH	+25-OH
	pmole/ min/ mg	
Fresh	516 ± 17	735 ± 6 (42%)
Frozen	515 ± 3	671 ± 28 (30%)
Heated	357 ± 4	438 ± 3 (23%)

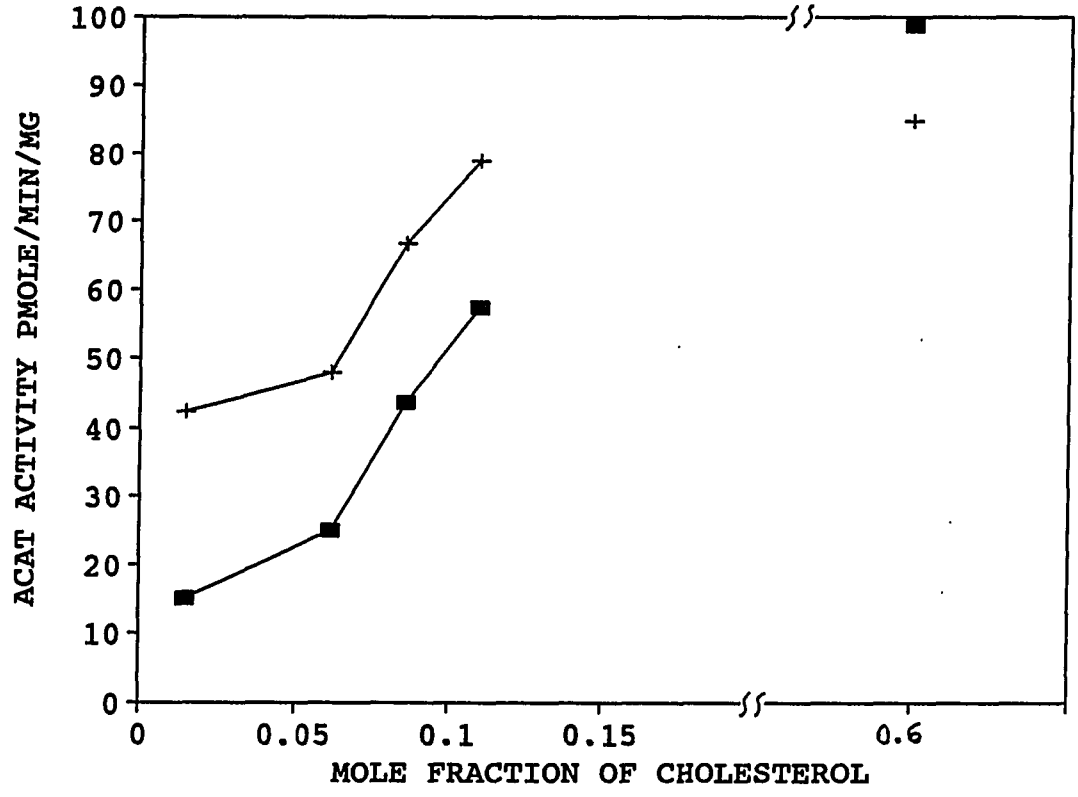
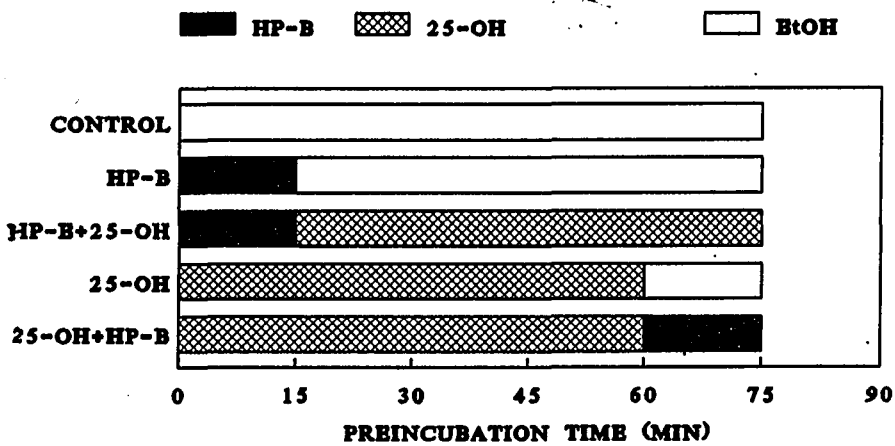


Figure 6. Effect of cholesterol concentration changes on stimulation of ACAT activity by 25-hydroxycholesterol. The reconstituted liposomes with varied concentrations of cholesterol were prepared according to method A described in Experimental. 25-OH (+) or an equal volume of ethanol (■) was added to the liposomes, and the mixtures were incubated 1 hr before ACAT activity was determined.



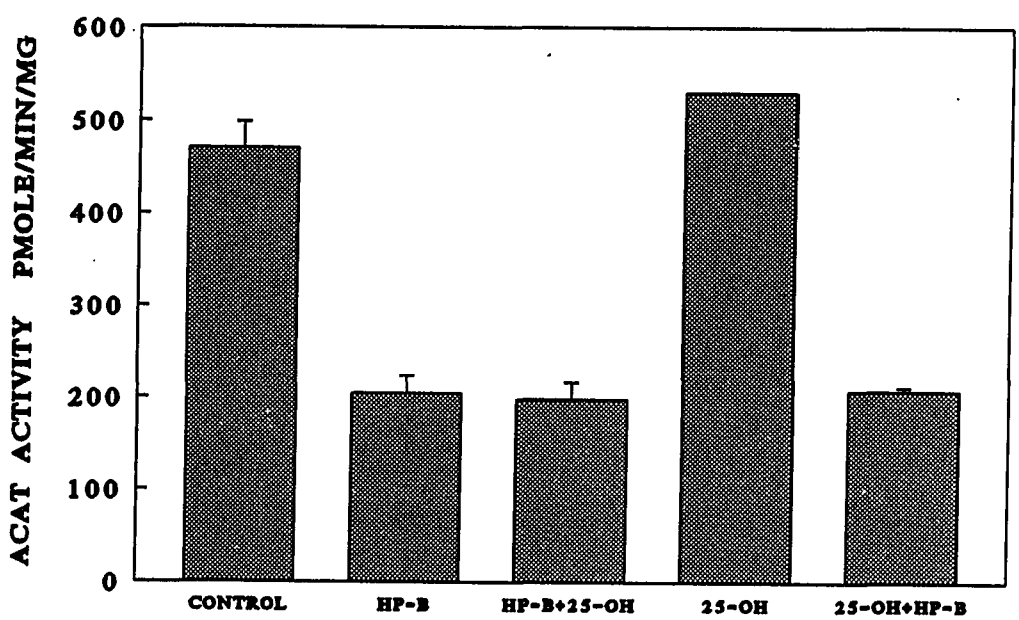


Figure 7. Effect of 25-hydroxycholesterol on HP-B treated ACAT activity. Microsomes were reacted with 25-OH or HP-B or both in the sequences shown in the upper panel. The concentrations of 25-OH and HP-B in the incubation mixtures were 31 μM.

level as in microsomes treated with HP-B alone. Nor was 25-OH able to activate ACAT that had reacted with HP-B.

C. Oxidation of model peptides and protein

1. Oxidation of glutathione by HP-B

Glutathione and glutathione disulfide were used as model peptides to study the effect of HP-B on cysteine and cystine residues. Glutathione alone was partly oxidized, presumably by autoxidation, in buffer A after 8 hr incubation. Table 6 shows that 40% of glutathione was detected as the disulfide form after incubation. The presence of 2 mM HP-B accelerated the formation of glutathione disulfide, and only 7.8% of glutathione was identified as the reduced form at the end of incubation. That is, HP-B converted 52% of cysteine residues to cystine. Under the same conditions, glutathione disulfide was very stable, and there was no detectable change due to the presence of HP-B (data not shown).

2. Oxidation of EPA by hydroperoxides

Methionine enkephalinamide (Met-EPA) and leucine enkephalinamide (Leu-EPA) are penta-peptides with amidated carboxyl terminals. The sequence of EPA is Tyr-Gly-Gly-Phe-X and the carboxyl terminal residue is either methionine amide or leucine amide. The EPA peptides were used to determine the effect of hydroperoxides on peptide methionine residues. The

Table 6. Oxidation of glutathione with HP-B. One portion of ethanol or 10 mM ethanolic HP-B was added to 4 portions of 1 mM GSH. The mixture was incubated for 8 hr at 37°C, and the reaction products were derivatized and analyzed by HPLC. The data represent the mean values of two determinations.

Treatment	Mole ratio GSH/GSSG
GSH only	3.05
GSH + HP-B	0.16

reactions of EPAs with cumene-HP were monitored with the aid of HPLC. Both EPA peptides were very stable in buffer A at 37°C for 24 hours. Leu-EPA was also resistant to the challenge of cumene-HP (Figure 8). But, when 5.9-fold excess of cumene-HP was added to Met-EPA, a new peptide peak eluted at 7.8 min was detected by HPLC after 35 min incubation. As shown in Figure 9, the production of new peptide accompanied by the disappearance of Met-EPA was time dependent. The peak at 31 min is cumene-HP, and the one at 24.5 min is presumably cumene hydroxide. The new peptide was collected from HPLC and characterized with FAB-mass spectroscopy. The spectra of Met-EPA and Met(O)-EPA are shown in Figure 10. The new peptide has a molecular weight of 588 which is higher than that of Met-EPA by 16. The mass difference suggests that the new peptide contains one more oxygen atom than Met-EPA. Moreover, the difference in reactivity of Leu-EPA and Met-EPA indicates that cumene-HP reacted specifically at the methionine residue. That is, cumene-HP oxidized the methionine to form methionine sulfoxide (Met(O)). Under the same conditions, HP-B oxidized Met-EPA at a much slower rate. Met(O)-EPA was observed in the reaction mixture of Met-EPA plus HP-B after 8 hr, but not 35 min. The addition of methanol accelerated the oxidation. In the presence of 20% methanol, Met(O)-EPA was formed within 1.5 hours (data not shown). Another oxidized derivative of methionine, methionine sulfone ($Met(O)_2$) was obtained from the reaction of Met(O)-EPA with an excess of performic acid.

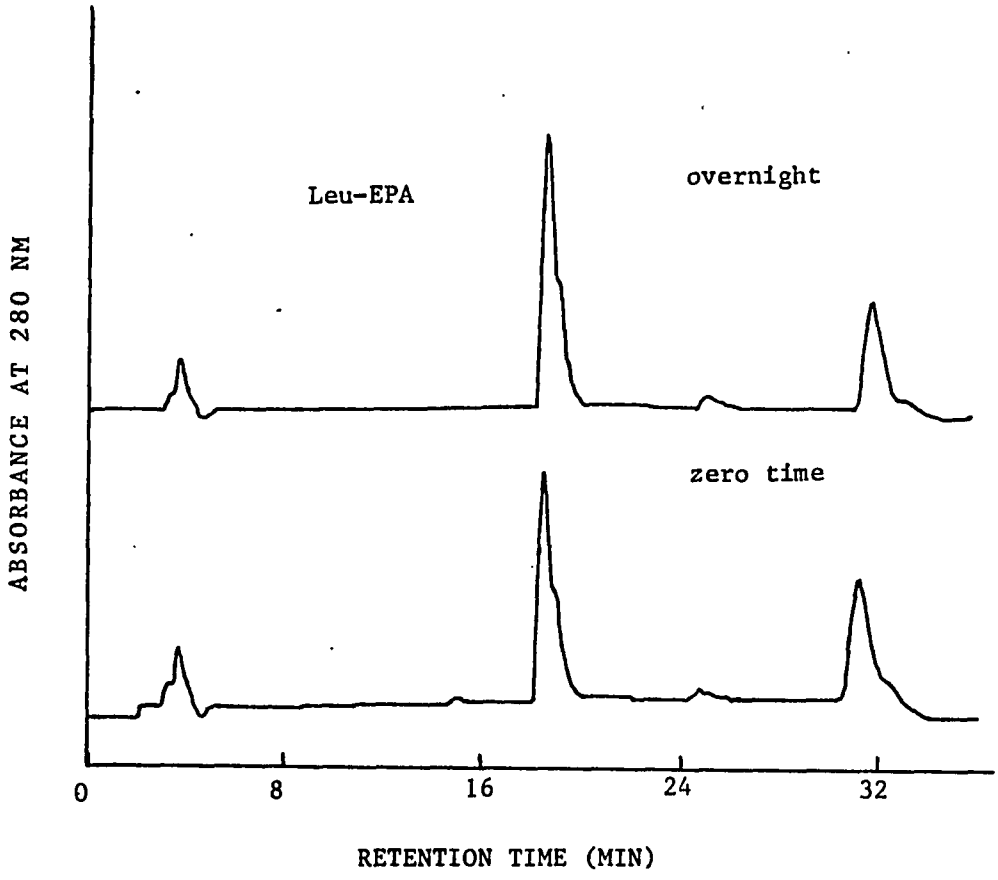
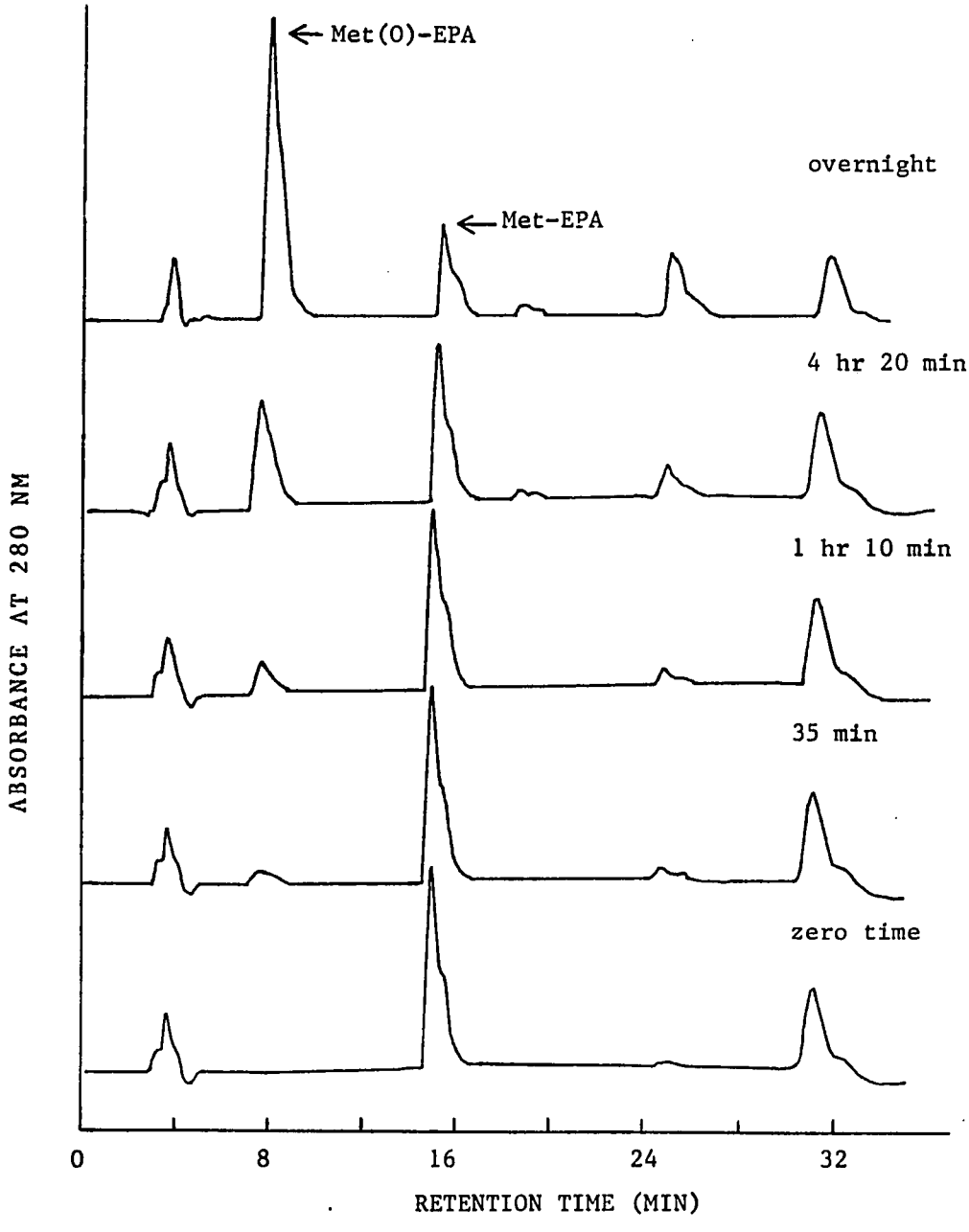


Figure 8. HPLC profile of Leu-EPA reacted with cumene-HP. Leu-EPA (1.7 mM) was incubated with 10 mM cumene-HP. At the indicated times, an aliquot of the reaction mixture was withdrawn and analyzed by HPLC. The HPLC conditions are described in Experimental, and the flow rate of mobile phase was 0.7 ml/min.

Figure 9. HPLC profile of Met-EPA reacted with cumene-HP. Met-EPA (1.7 mM) was incubated with 10 mM cumene-HP. At the indicated times, an aliquot of the reaction mixture was withdrawn and analyzed by HPLC. The HPLC conditions are described in Experimental, and the flow rate of mobile phase was 0.7 ml/min.


← Met(O)-EPA
overnight
←Met-EPA
4 hr 20 min
1 hr 10 min
35 min
zero time
ABSORBANCE AT 280 NM
0
8
16
24
32
RETENTION TIME (MIN)

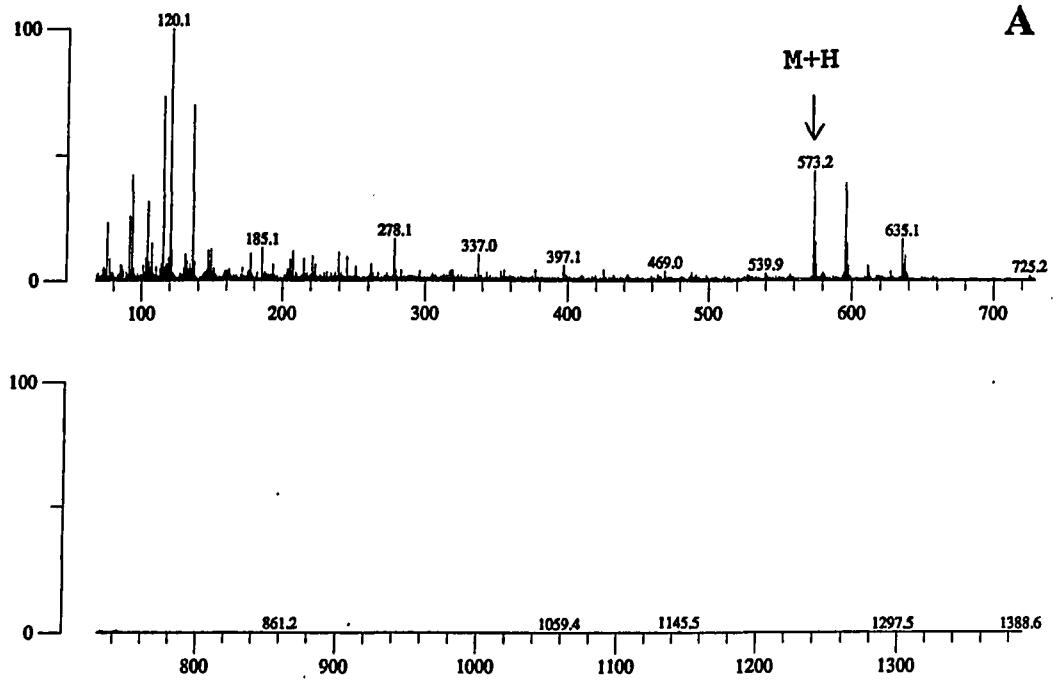


Figure 10. FAB-Mass spectra of (A). Met-EPA; (B). Met(O)-EPA; (C). Met$(O)_2$-EPA.

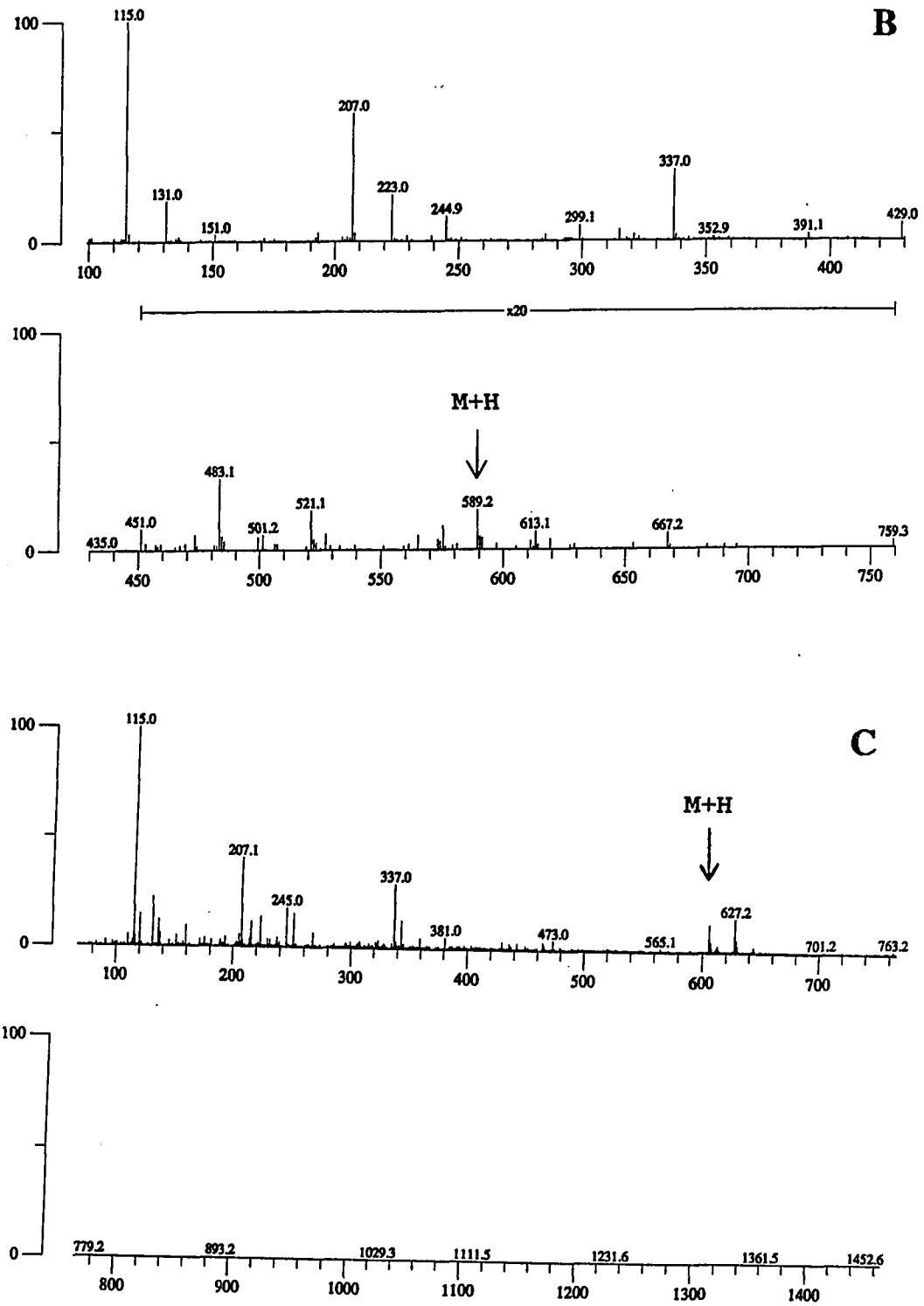

B
100
0
115.0
131.0
151.0
207.0
223.0
244.9
299.1
337.0
352.9
391.1
429.0
100
150
200
250
300
350
400
x20
M+H
483.1
521.1
589.2
451.0
435.0
501.2
613.1
667.2
759.3
450
500
550
600
650
700
750
C
115.0
207.1
245.0
337.0
381.0
473.0
565.1
627.2
701.2
763.2
779.2
893.2
1029.3
1111.5
1231.6
1361.5
1452.6
800
900
1000
1100
1200
1300
1400

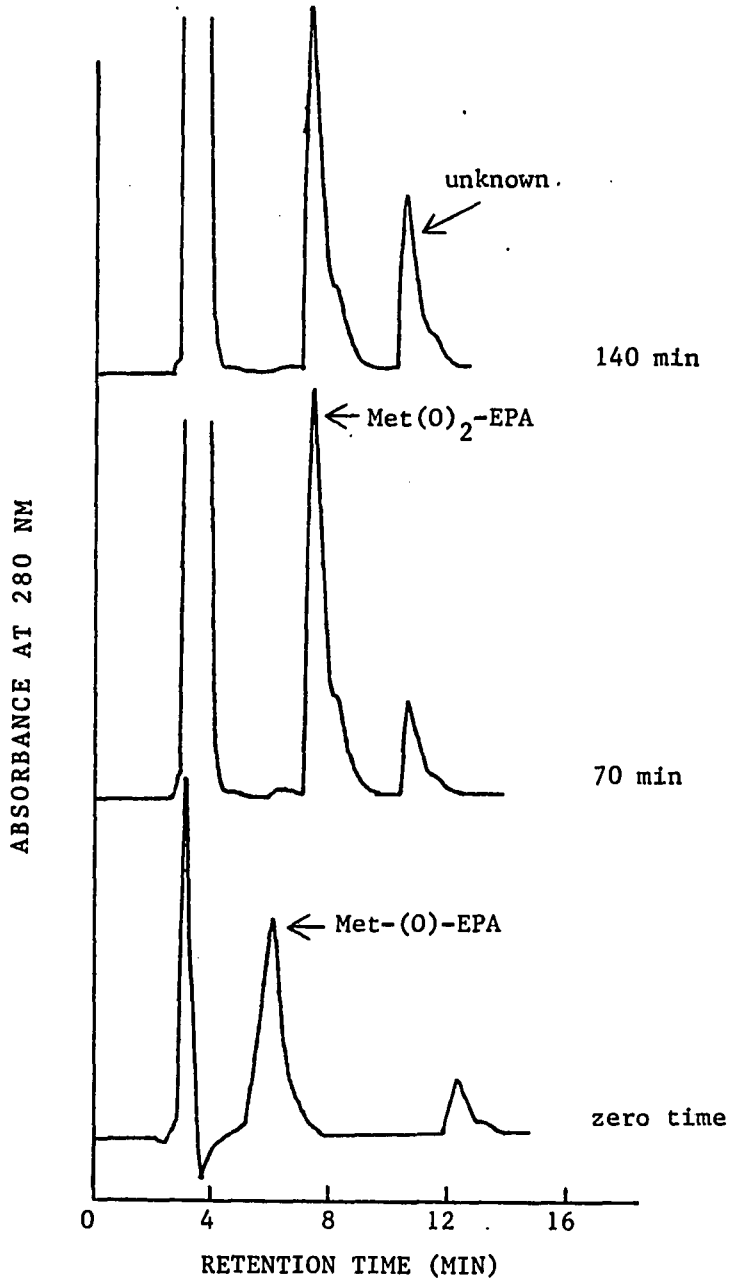
Figure 11 shows the HPLC profile of Met(O)-EPA and $Met(O)_2$-EPA. The retention times of Met(O)-EPA and $Met(O)_2$-EPA were 6 and 13 min. The structure of $Met(O)_2$-EPA was also confirmed by FAB-mass spectroscopy (Figure 10). Neither cumene-HP nor HP-B was able to further oxidize methionine sulfoxide to $Met(O)_2$ under our experimental conditions.

Attempts to reduce 1 mM Met(O)-EPA to Met-EPA with 100 mM reducing agents, DTT, DMP, $NaBH_4$, and NH_2OH, in one hour incubations at 37°C were not successful (data not shown). On the other hand, an overnight incubation of 13 μg Met(o)-EPA with 0.725 M MMA at 37°C completely restored the Met-EPA. The reduction of Met(O)-EPA by MMA is shown in Figure 12.

3. Oxidation of calmodulin by HP-B

Bovine brain calmodulin contains nine methionine residues and no cysteine residue. It was selected as a model protein to study the reaction of protein methionine residues with cholesterol hydroperoxides. Calmodulin was reacted with three different oxysterols, 7α-HP, HP-B, and Triol-B, for 30 min at 30°C. The reaction products were degraded by cyanogen bromide and characterized in 15% SDS gels. The results are presented in Figure 13. Calmodulin from commercial source was resolved as two bands with apparent molecular weights of 20 and 18.5 kDa. I suspect that these two bands correspond to calmodulin in two different conformations, with different amount of bound Ca^{++}. With large excess of CNBr, this protein was degraded

Figure 11. Reaction of Met(O)-EPA with an excess of performic acid. Met-EPA was reacted with an excess of H_2O_2 for 10 min before the performic acid was added (zero time). After the addition of performic acid, an aliquot was withdrawn at the indicated time and analyzed by HPLC. The flow rate of mobile phase was 0.85 ml/min.


unknown
140 min
Met(O)2-EPA
70 min
Met-(O)-EPA
zero time
ABSORBANCE AT 280 NM
0
4
8
12
16
RETENTION TIME (MIN)

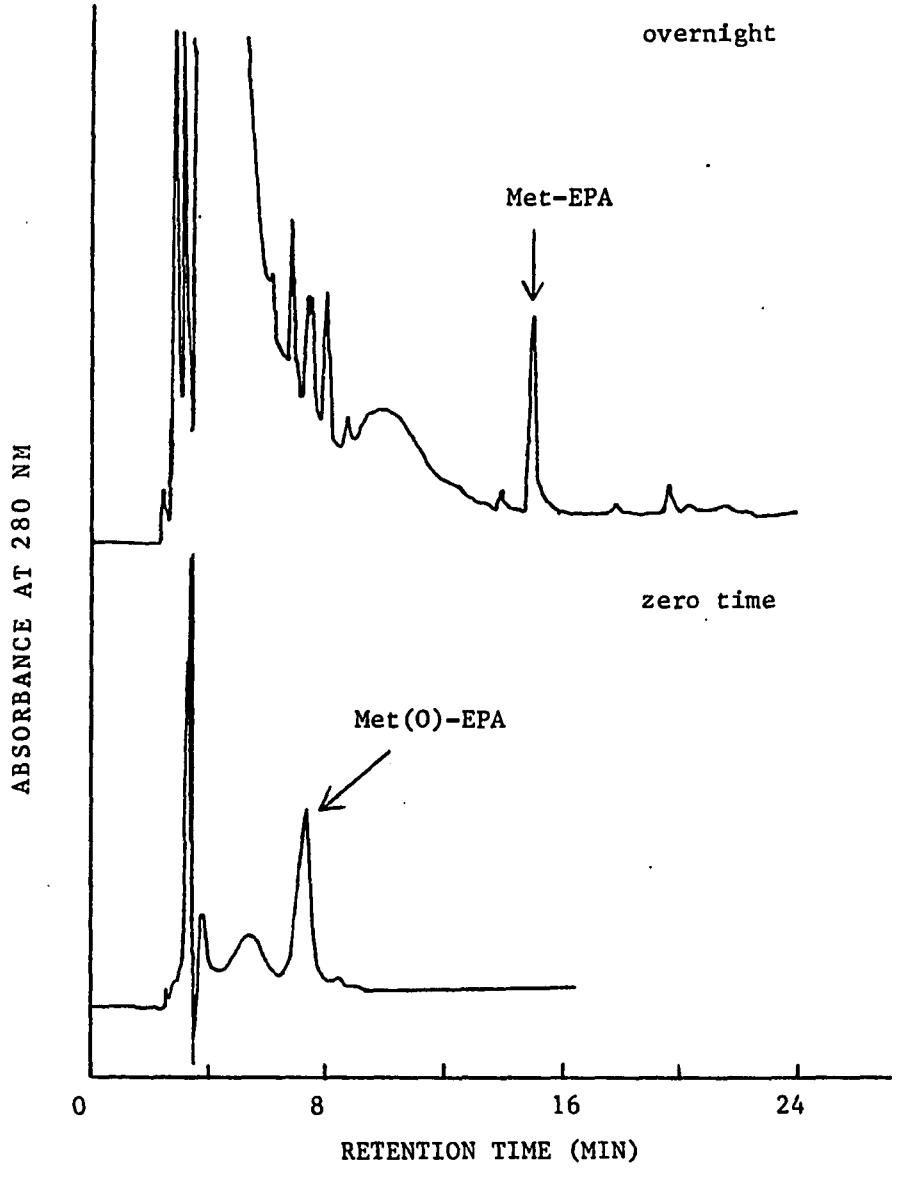
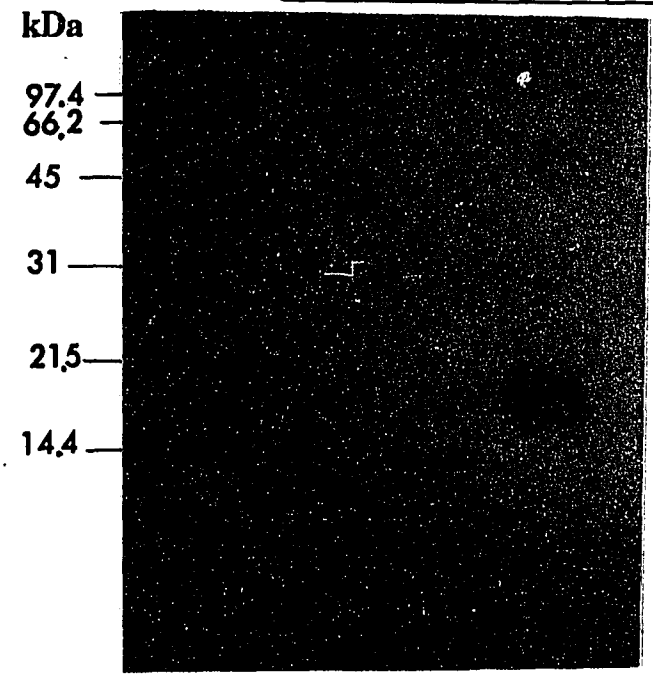


Figure 12. Reduction of Met(O)-EPA by MMA. HPLC profiles show the starting material, Met-EPA, and products from overnight reaction.

Figure 13. SDS-PAGE separation of CNBr-fragments of calmodulin. Calmodulin was treated with or without different oxysterols before CNBr degradation. The concentrations of CNBr used in panel A and panel B were 10 mg/ml and 1 mg/ml, respectively. The degradation products were separated in 15 % acrylamide gels and stained with Coomassie blue G. The molecular weight marker used in these two gels are the same. CaM: calmodulin.

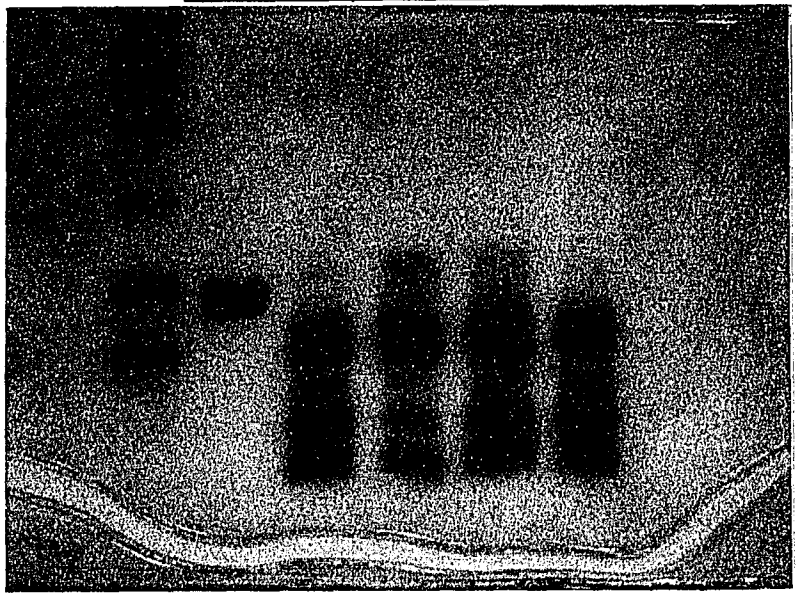
A

+	+	+	CaM
HP-B	-	-	oxysterol
+	+	-	CNBr



B

+	+	+	+	+	CaM
-	-	HP-B	7α-HP	Triol-B	oxysterol
-	+	+	+	+	CNBr



into three peptide fragments, one major band with apparent molecular weight 11.5 kDa and two minor bands of 15.5, and 8.5 kDa (Figure 13A). The HP-B treated calmodulin has a very different degradation pattern: two major fragments with apparent molecular weights of 16.5 and 15.5 kDa, one minor fragment of 11.5 KDa, and the 8.5 KDa band was almost undetectable. In another experiment (Figure 13B), control calmodulin was degraded by CNBr into three fragments, and the formation of the two small fragment was inhibited if calmodulin was preincubated with HP-B. In contrast, 7α-HP and Triol-B have no such effect, and the calmodulin degradation patterns after treatment with these compounds are very similar to that of the unreacted protein.

D. Effects of thiol-blocking and reducing agents on the activity of hydroperoxide-treated ACAT

1. Modification of ACAT with PMB and PAO

Microsomal ACAT was sensitive to the sulfhydryl-modifying reagent, PMB, which inhibited ACAT by 97% at a concentration of 1.0 mM (Table 7). Inhibition was partly reversible by DTT. At 1.3 mM, DTT restored 42-45% of the enzyme activity from microsomes exposed to 0.5 or 1.0 mM PMB. Microsomal ACAT was also sensitive to PAO, a reagent that reacts preferentially with vicinal sulfhydryl groups. As shown in Figure 14, ACAT

Table 7. Effect of PMB on ACAT activity. After 10 min of reaction with the indicated concentrations of PMB, microsomes were diluted 11-fold with buffer A with/without DTT and incubated for 10 min before ACAT assay. The final concentration of DTT was 1.3 mM. The percentage activity is shown in the parenthesis.

Treatment	ACAT activity	
	- DTT	+ DTT
	pmole/ min/ mg	
Control	555 (100)	534 (96)
0.5 mM PMB	110 (20)	345 (62)
1.0 mM PMB	18 (3)	264 (48)
2.0 mM PMB	15 (3)	136 (25)

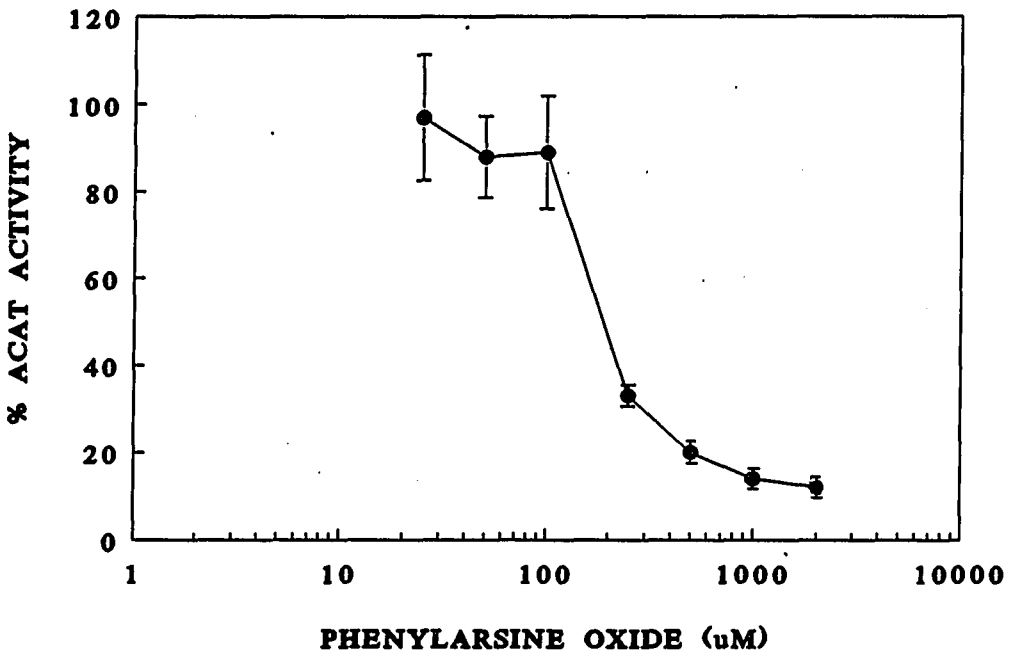


Figure 14. Effect of PAO on ACAT activity. Microsomes were incubated with the indicated concentrations of PAO for 30 min at 37°C. Microsomes were diluted 8-fold with buffer A before the ACAT assay.

activity gradually decreased when PAO concentrations were above 0.1 mM, and the IC_{50} of PAO was 180 μM. The specificity of PAO for reaction with vicinal sulfhydryls was examined by using two thiol reducing agents, DTT and DMP. Table 8 shows that DMP but not DTT was able to reverse the PAO reaction. At a concentration as low as 0.5 mM, DMP restored ACAT activity by 30%. But, increasing the concentration of DMP gave no further reactivation, and it was not possible to restore full activity.

Microsomes after reaction with PAO were further challenged by HP-B and the abilities of DMP to reactivate the doubly treated ACAT were determined. The result is shown in Table 9. More than 80% of the enzyme activity was inhibited by 0.5 mM PAO, and 20 % of the inhibition was reversed by 2 mM DMP. DMP also partially restored ACAT activity from HP-B-treated enzyme by 15%. Challenging microsomes with PAO, followed by HP-B did not decrease the enzyme activity further. DMP reactivated the doubly treated microsomal ACAT to the same extent as it did with microsomes reacted with PAO or HP-B alone. Thus, the inhibitory effects of PAO and HP-B were not additive. And the PAO-treated ACAT was protected from reaction of HP-B.

Table 8. Activation of PAO-inhibited ACAT activity by DMP and DTT. Microsomes (5 mg protein/ml) were incubated with 500 μM PAO for 30 min at 37°C, followed by a 16-fold dilution with buffer A containing the indicated concentrations of DMP or DTT. ACAT activities were determined 30 min after the dilution. Each value is the mean ± s.d. of two determinations.

Treatment	ACAT activity pmole/min/mg	percent of control
Control	176 ± 9	100
+ 1.0 mM DTT	164 ± 3	96
+ 1.0 mM DMP	192 ± 1	109
PAO	54 ± 1	30
+ 1.0 mM DTT	62 ± 1	35
+ 0.5 mM DMP	112 ± 2	63
+ 1.0 mM DMP	114 ± 1	64

Table 9. Partial protection of ACAT from HP-B by PAO. HP-B (0.1 mM) was added to intact or PAO-reacted microsomes. After 15 min, the mixtures were diluted 16-fold with buffer A with or without 2 mM DMP. ACAT activity was determined after 30 min incubation. Each value is the mean ± s.d. of two determinations.

Treatment	ACAT activity pmole/min/mg	percent of control
Control	309 ± 70	100
PAO	58 ± 1	19
HP-B	72 ± 1	23
PAO + HP-B	54 ± 5	18
DMP	267 ± 23	87
HP-B + DMP	115 ± 11	37
PAO + DMP	125 ± 24	40
PAO + HP-B + DMP	82 ± 4	36

2. Effect of reducing agents on HP-B-inactivated ACAT activity

The reversibility of HP-B-inactivated ACAT activity was studied by using reducing agents including DTT, $NaBH_4$, DMP, and MMA. Table 10 shows the result from DTT treatment. In the experiment of the upper panel, microsomes were diluted with buffer A containing DTT after the reaction of HP-B. The final concentration of DTT was 1.3 mM. With a brief incubation at 37°C, no appreciable increase in ACAT activity was detected. In the lower panel where higher concentrations (20-100 mM) and prolonged incubation (3 hr) was employed, ACAT activity was not restored by DTT. In fact, high concentrations of DTT caused 50% loss of activity in the control microsomes. On the other hand, $NaBH_4$, and DMP were able to restore a small amount of ACAT activity. In a short-term incubation, HP-B-reacted ACAT was activated by 10 to 200 mM $NaBH_4$ by 20% (Table 11). Table 12 shows the effect of 1-8 mM DMP on HP-B-treated ACAT activity. DMP at a concentration of 1 mM or greater slightly restored enzyme activity by 10%. But, considerable ACAT activity was lost at high concentrations of $NaBH_4$ or DMP.

N-Methyl-mercaptoacetamide was found to reactivate the ACAT activity very effectively (Table 13). While 0.1 mM HP-B suppressed 90% of the enzyme activity, incubation of the reacted microsomes with 10 mM MMA restored the enzyme activity to 55% of the control level. Also, an increase of 43% in the

Table 10. Effect of DTT on ACAT activity. In the upper panel, microsomes were reacted with or without 0.1 mM HP-B, followed by a 16-fold dilution with buffer A containing DTT. And, ACAT activity was determined after 30 min incubation. In the lower panel, microsomes reacted with or without HP-B were mixed with an equal volume of buffer A containing varied concentrations of DTT. The mixtures were incubated at 37°C for another 3 hr, followed by a 10-fold dilution before the ACAT assay.

Treatment	ACAT activity pmole/min/mg	percent of control
Control	-	-
+ 1.3 mM DTT	1203	100
HP-B	112	9
+ 1.3 mM DTT	171	14
Control	1430	100
+ 20 mM DTT	669	47
+ 50 mM DTT	691	48
+ 100 mM DTT	881	62
HP-B	699	49
+ 20 mM DTT	536	37
+ 50 mM DTT	589	41
+ 100 mM DTT	513	39

Table 11. Effect of $NaBH_4$ on ACAT activity. Microsomes reacted with 0.1 mM HP-B were mixed with an equal volume of buffer A containing the indicated concentrations of $NaBH_4$. The mixtures were incubated at 37°C for another 15 min. The microsomes were diluted 11-fold with buffer A for ACAT assay.

Treatment	ACAT activity pmole/min/mg	percent of control
Control	530	100
+ 10 mM $NaBH_4$	506	96
+ 20 mM $NaBH_4$	434	82
+ 100 mM $NaBH_4$	402	76
+ 200 mM $NaBH_4$	296	56
HP-B	112	21
+ 10 mM $NaBH_4$	211	40
+ 20 mM $NaBH_4$	210	40
+ 100 mM $NaBH_4$	194	37
+ 200 mM $NaBH_4$	182	34

Table 12. Effect of DMP on ACAT activity. Microsomes reacted with HP-B were diluted with an equal volume of buffer A containing the indicated concentrations of DMP for 30 min before an aliquot of 160 μl was withdrawn for ACAT assay. Each value is the mean ± s.d. of two determinations.

Treatment	ACAT activity pmole/min/mg	percent of control
Control	1262 ± 20	100
+ 4 mM DMP	1103 ± 27	87
HP-B	178 ± 6	14
+ 1 mM DMP	314 ± 13	25
+ 2 mM DMP	314 ± 24	25
+ 4 mM DMP	323 ± 29	26
+ 8 mM DMP	255 ± 7	26

Table 13. Effect of MMA on ACAT activity. Microsomes reacted with or without 0.1 mM HP-B were incubated with the indicated concentrations of MMA for 3 hr at 37°C. Microsomes were diluted 10-fold with buffer A before ACAT activity was measured.

Treatment	ACAT activity pmole/min/mg	percent of control
Control	1158	100
+ 10 mM MMA	1130	97
+ 100 mM MMA	800	69
HP-B	124	11
+ 10 mM MMA	644	55
+ 100 mM MMA	622	54

enzyme activity was detected after incubating the HP-B-reacted microsomes with 100 mM MMA. However, ACAT was not very stable in the presence of high concentrations of MMA. As shown in Table 13, ACAT activity in the control microsomes was reduced by 30% by 100 mM MMA. This reagent at 1 M abolished virtually all the enzyme activity (data not shown).

Finally, an experiment was performed to compare the reactivities of different reducing agents at the same concentration and in the same incubation time. Microsomes, intact or HP-B reacted, were incubated with 10 mM of reagents for 3 hr, and the ACAT activities were determined after proper dilution. As shown in Figure 15, $NaBH_4$ was the most effective reagent in restoring the ACAT activity and was the least damaging to the enzyme. N-Methyl-mercaptoacetamide was also quite effective. It restored 40% activity from HP-B reacted microsomes even though the intact microsomes lost 28% of activity. DTT and DMP appeared to be less useful reagents for ACAT activation because they are also inhibitors.

3. Effect of hydroxylamine and sodium chloride on ACAT activity

When HP-B reacted ACAT was incubated with NH_2OH, an appreciable degree of re-activation was observed. Figure 16 shows the effect of NH_2OH on ACAT activity at varied concentrations. HP-B inhibited 80% of ACAT activity, and the lost activity was partially recovered by NH_2OH. The activity

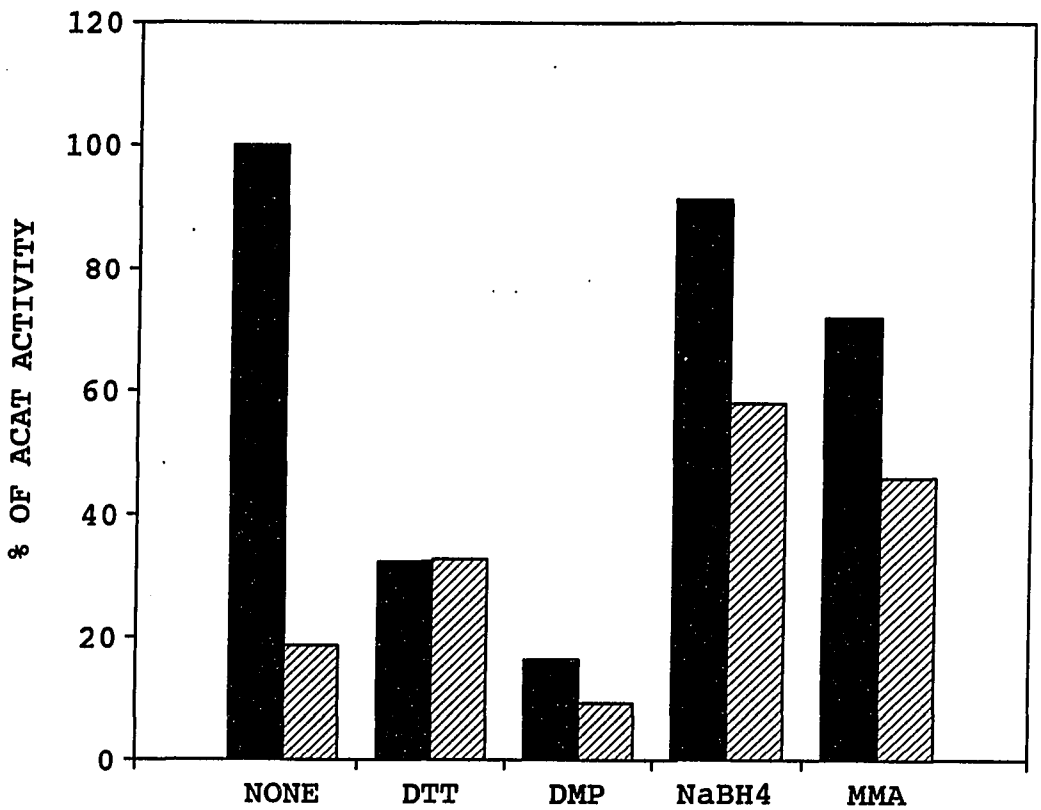


Figure 15. Microsomes were reacted with (hatched bar) or without (solid bar) 0.1 mM HP-B for 15 min before incubation with 10 mM of the indicated reducing agents. ACAT activity was determined after 3 hr incubation at 37°C and a 16-fold dilution with buffer A. Each value is the mean of two determinations.

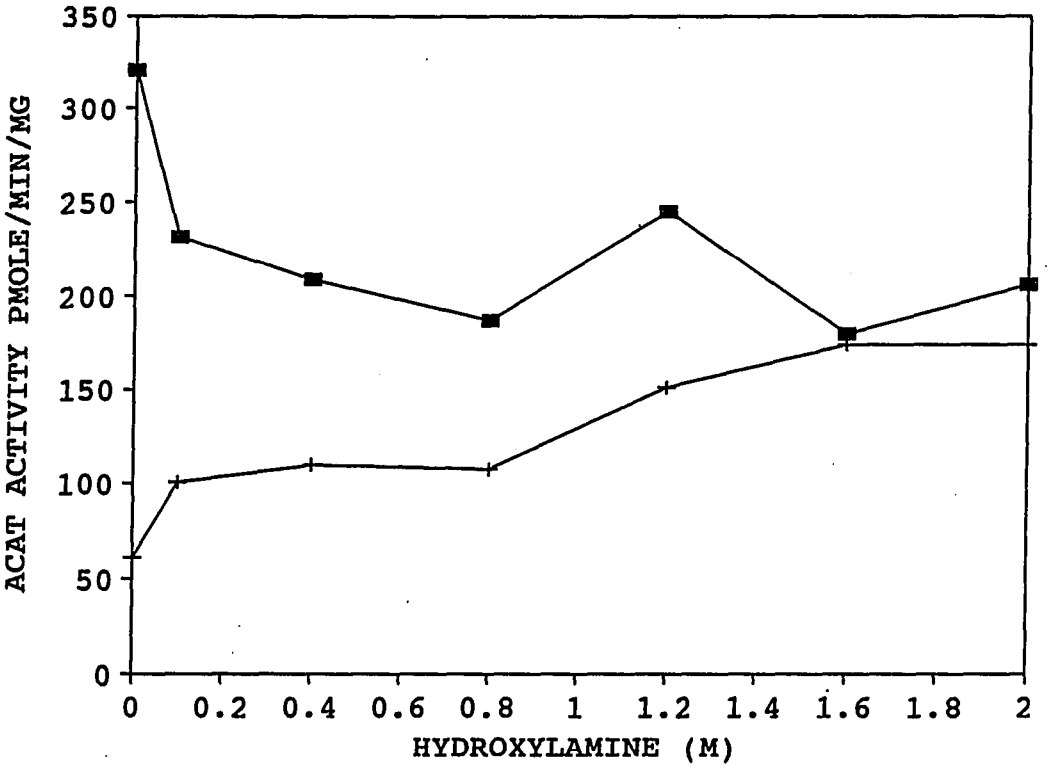


Figure 16. Effect of hydroxylamine on microsomal ACAT activity. NH_2OH at the indicated final concentrations was added to microsomes pre-treated with (+) or without (■) 100 μM HP-B. ACAT activity was measured after 30 min incubation. Each value is the mean of two determinations.

of HP-B-inhibited enzyme was almost triples by 2 M NH_2OH. On the other hand, intact ACAT lost approximately 30-45% activity in the incubation with NH_2OH alone. Because the NH_2OH solution was prepared by dissolving NH_2OH.HCl in water, followed by neutralizing with NaOH, it also contained a considerable amount of NaCl. Therefore, the effect of salt on ACAT activity was investigated. The result is shown in Table 14. While 3 M NaCl did not have a significant effect on intact ACAT, the salt caused a detectable activation of ACAT activity after HP-B treatment.

Table 14. Effect of NaCl on ACAT activity. Intact or HP-B reacted microsomes were incubated in buffer A containing the indicated concentrations of NaCl for 1 hr at room temperature. Microsomes were diluted 11-fold with buffer A before ACAT activity was determined. Each value is the mean ± s.d. of two determinations.

Treatment	ACAT activity pmole/min/mg	percent of control
Control	1496 ± 38	100
+ 3 M NaCl	1560 ± 22	104
HP-B	443 ± 10	30
+ 1 M NaCl	494 ± 2	33
+ 2 M NaCl	579 ± 22	39
+ 3 M NaCl	658 ± 6	44

V. DISCUSSION

The aim of the present study was to characterize the reaction of cholesterol hydroperoxides, especially HP-A and HP-B, with mouse liver acyl-CoA:cholesterol acyltransferase. Due to the difficulty in purifying this enzyme, I investigated the problem in the microsomal system. The enzyme activity is the only detectable index of the extent of reaction between ACAT and the tested compounds. And, our interpretation of the results is based on the presumption that ACAT is responsible for all cholesterol esterification in microsomal preparations. In addition, I took the following steps to assure that ACAT activity truly reflected the enzyme integrity. 1. Microsomes were carefully washed with buffer and centrifuged again. In this way, the possible contamination by soluble proteins, such as cholesteryl ester hydrolase, can be reduced. 2. ACAT activity was measured in a short time incubation. Microsomes contain acyl-CoA hydrolase, which effectively competes with ACAT for fatty acyl-CoA as substrate. A rapid assay prevents the complexity of results due to the activity of acyl-CoA hydrolase. 3. Bovine serum albumin was added to the assay solution. Both fatty acyl-CoA and fatty acid anion have detergent-like properties. The albumin acts as a reservoir for fatty acyl-CoA and fatty acid anion, preventing them from reaching concentrations at which their detergent properties become dominant (65).

How do HPs inhibit ACAT activity? Despite their structural similarity to cholesterol, HPs are unlikely to act as substrate analogs, competitively inhibiting the enzyme. I have observed that raising cholesterol concentrations in reconstituted liposomes did not protect ACAT from inhibition by HPs. As shown in Figure 2, even at a very high ratio (325) of cholesterol to HPs, the enzyme activity was still inhibited. In the experiment of Table 2, HP-B was added to preformed liposomes. Thus, ACAT should be able to bind cholesterol to the active site before it was exposed to HP-B. The fact that the binding of cholesterol did not prevent the interaction of HPs with ACAT suggests that HPs may react with enzyme at a region different from the active site.

It is also possible that HPs were esterified by ACAT to form the corresponding ester that did not co-elute with cholesteryl ester from silica gel columns. If this is true, I might have underestimated the ACAT activity and mis-interpreted the result as an inhibition of enzyme activity. But, this situation was unlikely to happen. In a study of the sterol substrate specificity of ACAT, Tavani et al (66) found that several 24-alkylsterols, including sitosterol, stigmasterol, ergosterol, and lanosterol, were poor substrates for the enzyme. Among a series of linear side chain analogs of cholesterol, cholesterol was the best substrate for ACAT, and analogs with either an increase or decrease in length of the side chain were not esterified as effectively. The author

concluded that the side chain is very important in the binding of substrate to the active site of ACAT. Accordingly, sterols with oxygenated side chains are unlikely to be esterified at an appreciable rate by ACAT. The HPs, with two oxygenated position on the side chain, should be even less likely to react.

With the aid of synthetic HPs analogs, I have demonstrated the importance of hydroperoxyl groups in ACAT inhibition. The result shown in Table 2 reveals that replacement of one or both hydroperoxyl groups with hydroxyl groups greatly diminishes the potency of HPs analogs. Table 3 shows that the dihydroperoxide, HP-B, reacted with ACAT more effectively than the mono-hydroperoxides, HP-B' and cumene-HP. HP-B inhibited the enzyme to a greater extent than the mono-hydroperoxides did, even though the latter substances were two-fold more concentrated than the former one. These observations imply that two spatially coordinated hydroperoxyl groups are required for maximal potency. I further tested this hypothesis with several synthetic dihydroperoxides whose structures are shown in Figure 4. All the synthetic dihydroperoxides are potent inhibitors of ACAT, and their potencies are greater than any of the mono-hydroperoxides I have tested. In a series of side chain analogs of HPs, the maximal ACAT inhibition was obtained when two hydroperoxyl groups were five carbons apart. Either insertion or deletion of one carbon in the side chain decreased the inhibitory

effect. These results imply that two hydroperoxyl groups probably interact with ACAT at two distinct positions. When the hydroperoxyl groups are 5 carbons apart, they approach these reactive sites most effectively. On the other hand, replacement of the tetra-cyclic structure of HPs with an indan structure did not reduce the inhibitory effect. Thus, while two hydroperoxyl groups directly interact with the enzyme, the ring structure may play a supportive role to provide sufficient hydrophobicity for the compounds to approach the enzyme.

Generally, hydroperoxides have high oxidation-reduction potentials and are considered as oxidants. The simplest hydroperoxide is hydrogen peroxide, which has been widely used for protein oxidation (67). Under suitable conditions, hydrogen peroxide reacts with several functional groups of proteins, such as thioether, sulfhydryl, disulfide, imidazole, and phenol. In acidic solution (pH<3), hydrogen peroxide almost exclusively oxidizes methionine (68), while under neutral and slightly alkaline conditions, cysteine, tryptophan, and other amino acids are also modified (69,70). The reactivity of hydrogen peroxide led me to investigate whether HPs would react with cysteine and methionine residues in proteins. Although HPs also possibly react with tryptophan and histidine, I did not investigate these two amino acids in the current study for two reasons: 1. Oxidation of tryptophan by hydroperoxide is pH dependent, and

the reaction is very slow at pH lower than 8 (69). I usually reacted the microsomal enzyme with HPs at pH 7.7. At this pH, oxidation of tryptophan is less likely to occur. 2. Oxidation of histidine with hydrogen peroxide under mild conditions, has only been demonstrated with the free amino acid. That hydrogen peroxide failed to modify poly-L-His and copoly-His-Glu peptides suggested that histidine residues in protein are more resistant to oxidation.

By using glutathione as a model peptide, I demonstrated the oxidation of cysteine residues by HPs (Table 6). Our results show that at pH 7.7, HPs oxidized sulfhydryl to disulfide and the latter functional group was not further oxidized. Little and O'Brien (71) have reported that glutathione disulfide and sulfonic acid were the major reaction products after oxidation of glutathione with hydrogen peroxide or linoleic acid hydroperoxide at pH 8.5 for 22 hr. If the reactivities of HPs are similar to those of hydrogen peroxide or linoleic acid hydroperoxide, HPs may be able to oxidize a sulfhydryl to a sulfonic acid under proper conditions. But this did not happen in our experiment. The success of the oxidizing the cysteine residue of glutathione by HPs suggests a possible reaction that may occur when microsomal enzymes were incubated with HP. But, oxidation of sulfhydryl groups in proteins is not exactly like that in small thiols, such as glutathione. Steric effects are important factors that need to be considered for oxidizing a

protein sulfhydryl. In some proteins, the steric factor may severely impede disulfide formation from hindered sulfhydryls. Upon oxidizing a hindered sulfhydryl with a strong oxidant, a sulfonic acid will be the main product instead of disulfide (72). Therefore, I expect that the oxidation of protein cysteine residues with HPs may produce disulfides and, possibly, sulfonic acids.

HPs also oxidize methionine. In the study with Met-enkephalinamide, I detected the oxidation of the methionine residue by HPs with HPLC and identified the reaction product as methionine sulfoxide with the aid of FAB-mass spectroscopy. To detect the oxidation of methionine residues of calmodulin by HP, I took a different approach. Cyanogen bromide has been widely used for protein fragmentation. This reagent specifically reacts with the thioether group of methionine, and alkylates it with a cyano group. The reactive sulfonium ion formed then cyclizes and results in peptide bond cleavage. However, if methionine is oxidized to methionine sulfoxide, this reaction cannot proceed. I took advantage of the different reactivities of methionine and methionine sulfoxide toward cyanogen bromide to detect the methionine oxidation in calmodulin by comparing the degradation patterns. Figure 13A shows that after a 30 min incubation, HP-B has oxidized certain methionine residues and caused a significant change in the cyanogen bromide fragmentation. A new peptide fragment with apparent molecular weight 16.5 kDa was derived from the

HP-B-reacted calmodulin. The size of the new fragment is only slightly smaller than the intact calmodulin, molecular weight 18.5 KDa, by 2 kDa. Thus, the new peptide has most of the structure of calmodulin with only approximately 20 amino acids cut off. Walsh et al (61) reported that among 9 methionine residues of calmodulin, Met-71, -72, -76, and possibly -109 are exposed on the surface and most susceptible to oxidation. The first three methionine residues are located in the central helical region of the protein. The new peptide I detected retains the central portion of calmodulin. This result suggests that HP-B very likely oxidized these four methionine residues. In contrast, 7α-HP did not change the protein degradation pattern. Thus, this hydroperoxide has no reaction with these methionine residues. Interestingly, HPs but not 7α-HP at lower concentrations than those used in these experiments are effective inhibitors of calmodulin activity in the cyclic-AMP phosphodiesterase assay (10). Our current result indicates that oxidation of methionine residues at the central helix of calmodulin contributes to the protein inactivation by HPs.

The result of the oxidation of cysteine and methionine residues in the model systems led me to suspect such reactions also occurred when ACAT was incubated with HPs. However, it is impossible to directly detect these reactions without a purified protein. I then tried to re-activate the HP-reacted ACAT with several reagents, expecting that the ability to

reverse an unknown protein modification by a specific procedure would provide useful information in characterizing the nature of the modifying reaction. Four mild reducing agents, including DTT, DMP, MMA, and $NaBH_4$, were used for this purpose.

Dithiothreitol, known as Cleland's reagent, has been used commonly in the reduction of protein disulfide bonds (73). A 30-100 fold excess of DTT was able to reduce exposed disulfide bonds within 30 min at pH 8.1 (74). The activity of DMP is similar to that of DTT. In addition, Dimercaptopropanol is known as anti-lewisite for its potency in dissociation of dithioarsenite (75). Sodium borohydride also reduces disulfide bonds (76), but this reagent is expected only to react with exposed disulfides. For example, borohydride selectively reduced one of six disulfides of trypsinogen even at very high concentration, 100 mM (77). Dedman (78) compared the reactivity of DMP and potassium borohydride in the reduction of methionine sulfoxide in corticotropin. He found that DMP reduced the hormone after 90 hr incubation but borohydride failed to do so. MMA reduces disulfide bonds as well. It has been reported as most effective in reduction of methionine sulfoxide and DTT was slightly less effective than MMA (55). Under neutral conditions, MMA or DTT at 0.7-2.8 M completely reduced methionine sulfoxide to methionine in peptides and proteins at 37°C in 12-24 hr. Generally, the reduction of methionine

sulfoxide requires a higher concentration of the reducing agent, longer reaction time, and probably a higher temperature than does the reduction of disulfide bond. In some cases, the choice of a reducing agent for reduction of methionine sulfoxide in a particular protein can be critical. For example, while mercaptoethanol was effective in reducing a methionine sulfoxide in yeast invertase; DTT and cysteine had no effect (79).

All reducing agents, I tested restored ACAT activity to a certain extent under proper conditions (Table 10-13 and Figure 15). After a 3 hr incubation, both $NaBH_4$ and MMA restored more than 50% of the enzyme activity from HP-B reacted ACAT. And, the effect of $NaBH_4$ was detectable even in a 15 min incubation (Table 11). DMP at 1 mM slightly activated the HP-B reacted ACAT in a 30 min incubation (Table 12), but at a higher concentration (10 mM) and for longer incubation time, it greatly inhibited the enzyme activity (Figure 15). At 1.3 mM, DTT did not restored ACAT activity in 30 min incubation. At 10 mM for 3 hr incubation, DTT restored a detectable amount of HP-B treated ACAT activity but it also greatly decreased the unreacted enzyme activity (Figure 15). The fact that all these four reducing agents were able to partially restore ACAT activity indicates that reduction of either disulfide bonds or methionine sulfoxides or both are possibly involved. Under our experimental conditions, in terms of the concentrations of reagents and incubation time, I expected that disulfide

reduction would occur more readily than the sulfoxide reduction. $NaBH_4$ was the most effective reducing agent to reactivate the HP-B-treated enzyme. This reducing agent has been shown to react primarily with exposed disulfide bonds and not with hindered ones. Thus, if it is indeed a disulfide reduction, the disulfide bond should be easily accessible to the medium and to the reducing agents. The differences in oxidation-reduction potentials of borohydride and thiols may account for their effectiveness to reduce the disulfide bond. On the other hand, because none of the reducing agents could completely restore the enzyme activity, it is possible that other types of protein modification also occur. Interestingly, DTT and DMP but not MMA or $NaBH_4$, strongly inhibited the enzyme at 10 mM, and the former two reagents are both dithiols. At this moment, I have no good explanation for this result.

A general survey of the effects of protein modification reagents on ACAT activity was performed by Kinnuene et al. (18). Their results showed that rabbit liver and aortic ACAT were sensitive to the reagents that modify histidyl, sulfhydryl and arginyl residues. The effect of sulfhydryl modification with PMB on ACAT activity was further studied. The result suggested that at least two sulfhydryl groups influence ACAT activity: one is at or close to the inhibitory CoA binding site and the other is necessary for cholesteryl ester formation. Our study with mouse liver ACAT shows that

this enzyme also sensitive to PMB modification but in slightly different ways. In one experiment, microsomes were incubated with 1 mM PMB for exactly 1 min, followed by a 10-fold dilution with buffer A with or without 1.3 mM DTT. I found that ACAT activity was inhibited to 13% of control, and DTT restored the activity to 70% of control (data not shown). Under similar conditions, the rabbit enzyme was completely inhibited by PMB, and the inhibition could be fully reversed by DTT. In another experiment, I increased the PMB reaction time to 10 min. By doing this, I found that mouse ACAT was inhibited by this thiol-blocking agent in a dose dependent way (Table 7) similar to what was reported for the rabbit enzyme. However, DTT still failed to restore all the enzyme activity. I performed the PMB reaction and ACAT assay in Tris buffer containing 1 mM EDTA, whereas Kinnuene did the experiment in phosphate buffer without EDTA. I suspect that the buffer system may have some effects on the PMB reaction. On the other hand, prolonged incubation of ACAT in the presence of PMB may have caused a certain degree of protein denaturation. An hypothesis of how PMB induces protein denaturation has been discussed by Torchinsky (80). The author suggested that if a sulfhydryl modified by PMB is participating in an interaction with other local residues to maintain the proper structure of the protein, such modification will disturb the interaction and subsequently destabilize the protein conformation. Because the change in conformation may not

occur instantaneously after the modification, the protein denaturation is therefore sluggish and time dependent. Delay in reversing the PMB modification may cause a higher degree of protein conformational change, which then requires a longer time to be re-natured. The differences in PMB reactivity and DTT reversibility may also imply that ACAT from different sources are not identical. It is known that the reactivity of sulfhydryl groups is influenced by other nearby functional groups. For example, charged functional groups will either promote or hinder the approach of ionized reagents to sulfhydryl groups. And, the neighboring residues can alter the degree of ionization of sulfhydryl groups. Thus, sulfhydryl groups of the mouse and rabbit enzymes may be located in different environments that cause the difference in their reactivity.

ACAT activity was sensitive to another sulfhydryl modification agent, PAO. This reagent inhibited the enzyme in a concentration dependent way, and the IC_{50} was 180 μM. PAO is highly specific for modifying vicinal sulfhydryl groups (81). It has been used to probe enzymes with proximate sulfhydryl groups (20,82). The fact that ACAT is inhibited by a low concentration (0.1 to 1.0 mM) of PAO suggests the presence of a pair of vicinal sulfhydryl groups that are important for the enzyme activity. I further tested the specificity of the PAO reaction with two dithiol reducing agents, DTT and DMP. It has been shown that upon reaction

with arsenoxide, DMP forms a five-member dithioarsenite ring that is more stable than the seven-member ring produced from DTT (83). Therefore, DMP is specifically used to reverse the PAO modification. Our result in Table 8 shows that PAO inhibition was partially reversed by DMP, but not DTT. This result confirms that the modification by PAO was at vicinal sulfhydryl groups, and the modification at these groups causes ACAT inhibition.

The experiments showing restoration of ACAT activity by reducing agents indicate that HPs probably oxidized ACAT sulfhydryl groups. If that is true, modification of sulfhydryl groups with a thiol-blocking agent should be able to prevent the reaction with HP. To evaluate this hypothesis, I incubated microsomes with PAO before the reaction of HP-B, and I determined the enzyme activity after reversing the thiol modification. It is shown that DMP was able to restore 15% or 20% of the ACAT activity from the PAO or HP-B reacted enzyme (Table 9). If PAO and HP-B reversibly modified ACAT at distinct sites, DMP should be able to restore 35% (15% plus 20%) of the enzyme activity. But, this was not true. Thus, HP-B and PAO must have common modifying sites, the vicinal sulfhydryl groups. On the other hand, the sequential reaction with HP-B did not further decrease the PAO-treated ACAT activity. And the degree of reactivation by DMP from PAO-reacted or doubly reacted enzyme are almost the same (20% vs 18%). These results indicate that PAO has effectively

blocked the reaction of HP-B, and reaction at such 'common modification site' by either PAO or HP-B greatly decreased ACAT activity.

Summarizing the results in characterization of ACAT inhibition by HP, I have the following conclusions: 1. HPs are not competitive inhibitors of ACAT, because increasing cholesterol concentration did not affect the inhibitory effect. 2. HPs oxidize vicinal sulfhydryl groups ACAT to form disulfide bond(s), which was reversible by reducing agents including $NaBH_4$ and MMA. Oxidation of such vicinal sulfhydryl groups greatly decreases the enzyme activity. 3. HPs may also react with non-cysteine residues, such as methionine or others that can not be protected by PAO, and these reactions are more resistant to reducing agents. Modification at these residues has less effect on ACAT activity.

The reaction of a pair of vicinal cysteine residues with PAO produces a cyclic dithioarsenite, which can be used to estimate the distance between two cysteine sulfur atoms. If the reaction of PAO does not cause significant protein conformational change, the distance of the two sulfur atoms before reaction will be similar to that in the cyclic dithioarsenite. The distance between two sulfur atoms in the arsenite has been calculated as 3.50-3.62 Å (84). This distance is much longer than a disulfide bond that is about 2.0 Å (85). How can HPs oxidize these sulfhydryl groups to form a disulfide bond? HPs may need to induce a protein

conformational change in such a way that two sulfhydryl groups come into a proximity that permits the disulfide formation. HPs may change enzyme conformation by modifying an amino acid residue near the sulfhydryl groups. After reacting with a near-by residue, HPs are able to effectively oxidize the sulfhydryl groups and form a disulfide bond. Summarizing this idea, I further propose a two-step modification model of the interaction of HPs with ACAT. The model is illustrated in Figure 17. When HP-A or HP-B approaches the vicinal sulfhydryl groups, it first modifies a near-by residue with one of the hydroperoxyl groups. This modification results in a protein conformational change. Then, HPs oxidize the two sulfhydryl groups to form disulfide bond. In this model, I presume that oxidation of a pair of vicinal sulfhydryl groups causes ACAT inhibition by a magnitude much greater than modification of other residues. This two-step modification model can also explain the observation that HP-A and HP-B were more potent than the mono-hydroperoxides, HP-A' and HP-B' (Table 2 and 3), because the complete reaction requires two hydroperoxyl groups. HP-A' or HP-B' may either react with the near-by residue, causing a conformational change without significantly affecting the enzyme activity or modify one of the sulfhydryl groups to sulfonic acid, if possible, and change the enzyme activity.

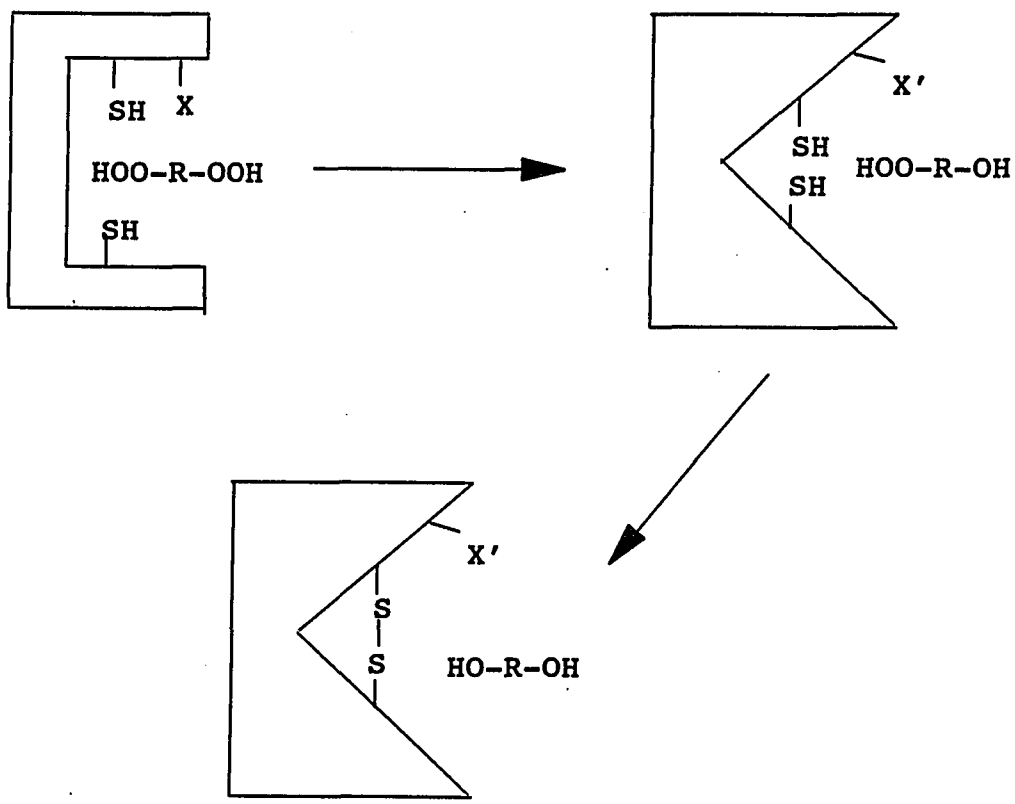


Figure 17. A proposed scheme of the ACAT inhibition by HP.

Although in our model, the vicinal sulfhydryl groups are very important for ACAT activity, I do not imply that they are directly involved in enzyme catalytic function. There are several examples of enzymes sensitive to sulfhydryl modification but not having cysteine residues involved in the catalytic mechanisms. One of these is lecithin cholesterol acyltransferase (LCAT), which catalyzes a reaction similar to the ACAT reaction. Researchers found that there are two cysteine residues (86) (Cys^{31} and Cys^{184}) near the serine residue at position 181, which was proposed to form an oxyester with an acyl group from phosphatidylcholine (87). Modification of either cysteine residue decreased the enzyme activity (86,87,88,89). Accordingly, it has been proposed that the two sulfhydryl groups participate in the catalytic mechanism by forming an S-acyl intermediate. However, this hypothesis was disproven because a mutant LCAT with the two cysteines at positions 31 and 184 replaced by glycines was found to be fully active (90). By analogy with LCAT, I suggest the vicinal sulfhydryl groups of ACAT may not have catalytic functions. Modification of these groups will either cause a steric effect that interferes with the substrate binding, or a conformational change which deactivates the enzyme.

In the study of the effect of cholesterol on ACAT inhibition, I noticed that ACAT activity was dependent on cholesterol concentration in a special way. Plotting enzyme

activity against cholesterol mole fraction in cholesterol/ phosphatidylcholine liposomes, I obtained a sigmoidal curve (Figure 1). (Due to the lack of data points between 0.01 to 0.1 mole fraction of cholesterol, the sigmoidal curvature could not be seen in Figure 2). Similar kinetic data have also been reported with reconstituted ACAT from Chinese hamster ovary cells (91,92). However the author did not discuss such observations in detail. Generally, the sigmoidicity of an activity-concentration plot suggests the presence of a cooperative interaction between binding sites of the enzyme. There are two types of cooperative interaction, positive and negative. Positive cooperative interaction means that the binding of one substrate molecule to the enzyme results in an increase in the affinity of the enzyme for the next substrates. Due to the lack of purified enzyme, it is not known whether ACAT has more than one binding site or contains more than one subunit. The kinetic results I observed imply that ACAT may be an allosteric enzyme which contains more than one subunit or binding site. In order to further investigate the cooperative interaction of ACAT active sites, I re-plotted the enzyme activity vs cholesterol concentration in Figure 1 into Hill coordinates (93), log [v/(Vmax-v)]; log [S], where v is the enzyme activity, Vmax is the maximal activity, and [S] is substrate concentration. The Hill coefficient, N_H, is the slope of the tangent of the plot at the chosen point. N_H reflects the nature of cooperative

interaction. When N_H is larger than unity it means a positive cooperative effect, and vice versa (94). As shown in Figure 18, the N_H of ACAT is less than unity when [S] is lower than 0.04 mole fraction, and N_H increases and becomes larger than unity when [S] is equal to or greater than 0.04 mole fraction. This result indicates ACAT may proceed via a complicated cooperative interaction between binding sites. For example, if ACAT contains three binding sites, binding the first cholesterol molecule will decrease the affinity of the enzyme for the next substrate molecule. But, when the second site is occupied, the enzyme will have a much greater affinity for cholesterol.

The idea that ACAT may be an allosteric enzyme leads us to ask if there is a physiological effector of this enzyme? 25-Hydroxycholesterol has been found to be a potent regulator of intracellular cholesterol metabolism. This compound decreases cholesterol synthesis by inhibiting HMG-CoA reductase activity (95,96). On the other hand, it increases cholesteryl ester formation in fibroblasts (97), hepatocytes (98), and intestinal cells (54). Drevon et al. (99) reported that 25-OH stimulated ACAT activity in both intact hepatocytes and microsomes, and the stimulation did not require protein synthesis. In the study with intestinal cells, Field and Mathur (54) demonstrated that the stimulation was inversely related to microsomal cholesterol content. At cholesterol concentrations below saturation, 25-OH has a stimulatory

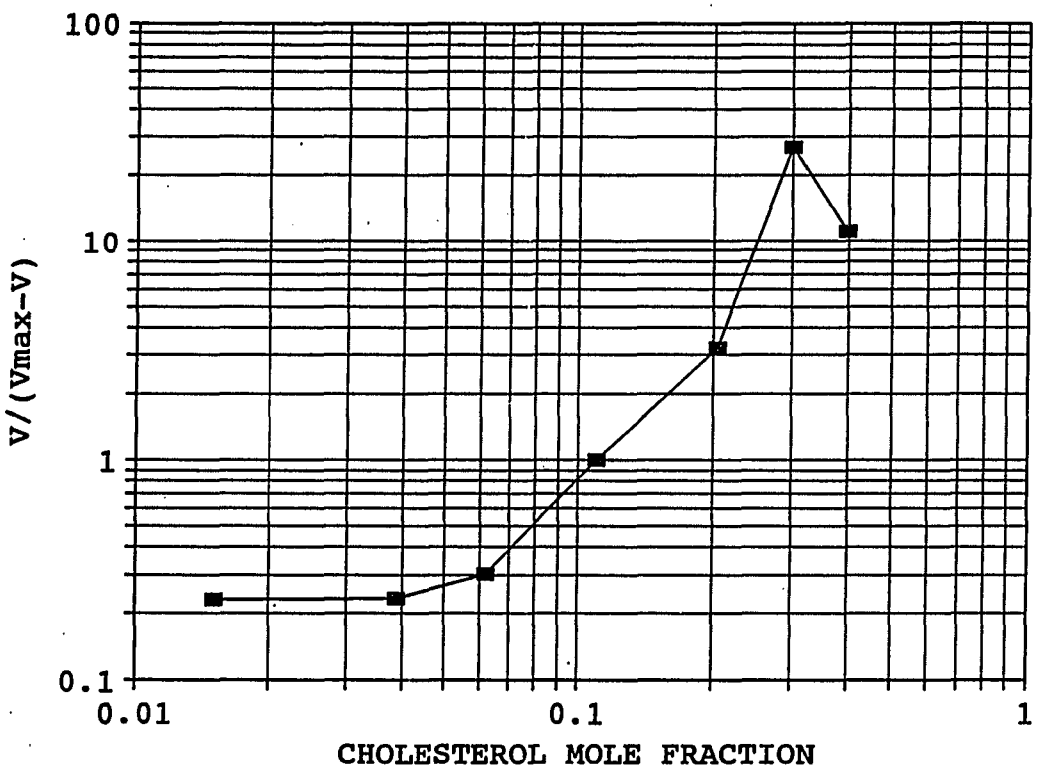


Figure 18. Hill plot of the effect of cholesterol concentration on ACAT activity. The experiment is the same as Figure 1. Only the data from control group (no HP-A was added) were replotted.

effect. And, if ACAT is saturated with respect to cholesterol, the oxysterol has no effect. The authors suggested two mechanisms for the effect of 25-OH: 1. 25-OH may interact with the microsomal membrane and increase the cholesterol in the ACAT substrate pool. 2. 25-OH may react with ACAT at a regulatory site that is shielded by an excess of cholesterol. Up to this time, there is no evidence to exclude either mechanism. My study with reconstituted liposomes has demonstrated the first time such stimulation can be reproduced in an artificial lipid environment. As shown in Figure 6, the effect of 25-OH was observed only at cholesterol concentrations less than saturation. Our observation not only indicates that 25-OH is able to regulate ACAT independent of its original lipid environment but also suggests that if any other factors are required for ACAT stimulation, they are also extractable by deoxycholate and are stable enough to be reconstituted. In separate experiments, I further demonstrated that the effect of 25-OH on microsomal ACAT was time dependent (Figure 5). And the effect was diminished by heat or a process of freezing and thawing (Table 5). However, our efforts to characterize the ACAT stimulation did not enable us to disprove either of the two possible mechanisms. In liposomes, 25-OH may interact with lipid vesicles in a way that causes cholesterol transfer from one liposome to another. Time-dependent activation can be explained as the time-dependent cholesterol transfer. Heat and cold treatments both

can cause membrane lipid re-arrangements which subsequently may affect the availability of cholesterol to the enzyme. Alternatively, I can explain that the time-dependent ACAT activation is due to a slow protein conformational change induced by an allosteric effector, 25-OH. It has been discussed by Kurganov (100) that the magnitude of the effect of an effector on enzyme activity depends on the time of contact of the enzyme with the effector. This is because the conformational change induced by the effector does not proceed instantaneously but at a finite rate. On the other hand, the heat or freezing treatment may change protein conformations that also affects the affinity of the effector binding site.

From the result shown in Figure 7, I conclude that if there is a regulatory site for 25-OH, the binding of oxysterol has no effect on ACAT inhibition by HPs. Thus, HPs probably attacks ACAT at a site other than an effector binding site.

VI. CONCLUSION

In the present study, I have demonstrated that 20(R and S)-20,25-dihydroperoxy-5-cholesten-3ß-ol (HPs) are the two most potent ACAT inhibitory oxysterols isolated from cholesterol autoxidation products. The side chain structure of HPs are critical for the potency, but a replacement of the tetra-cyclic structure with an indan did not affect their activities. HPs inhibit ACAT through chemical modification rather than competition with cholesterol as enzyme substrates. Experiments with reconstituted liposomes showed that cholesterol at an extremely high ratio to HPs failed to protect ACAT from inhibition. With model peptides and protein, including enkephalinamides, glutathione, and calmodulin, I have demonstrated that HPs oxidize protein sulfhydryl and thioether groups and the reaction products are disulfide and methionine sulfoxide. Without the purified ACAT, it is not possible for us to directly detect the reaction of HPs with the enzyme. However, the fact that reducing agents, DTT, DMP, MMA, and $NaBH_4$ partially restored the HP-B-treated ACAT activity suggests that cysteine and methionine oxidation possibly occurred. ACAT was sensitive to PMB and PAO, indicating that sulfhydryl, specifically, vicinal sulfhydryl groups are important for the enzyme activity. The reactions of these two thiol-modifying reagents with ACAT were partially reversed by DTT and DMP. Dimercaptopropanol

restored ACAT activity from PAO and HP-B doubly treated microsomes as effectively as from the PAO-treated microsomes. Thus, the major effect of HPs on ACAT activity is likely due to the reaction with vicinal sulfhydryl groups. Summarizing all the observations, I propose a scheme of sequential modification to explain the inhibitory effect of HPs on ACAT. That is, HPs first oxidize an unknown residue causing a protein conformational change without significantly affecting the enzyme activity. Then, HPs oxidize a pair of vicinal sulfhydryl groups to form a disulfide bond that results in a great loss in ACAT activity.

In addition, two unique features of ACAT activity have been observed in the present study. 1. ACAT activity shows a sigmoidal response to an increase in cholesterol concentration. This result implies that ACAT may have multiple cholesterol binding sites that interact with each other cooperatively. 2. 25-Hydroxycholesterol was able to stimulate ACAT activity even though the enzyme was isolated from its original lipid environment and reconstituted in to liposomes.

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